Production of *Monascus* pigment by solid state culture on adlay

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Submitted for the degree of Doctor of Philosophy

Heriot-Watt University

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November 2012

ABSTRACT

Angkak is a natural red colourant which is usually made by culturing *Monascus spp*. on cooked rice. Changing from rice to other raw materials which are richer in nutrients could increase pigment yield. Adlay (Chinese pearl barley) has been used as a health food supplement and in traditional Chinese medicine and could be used as a new substrate for angkak production. Evidence also exists that mycotoxin formation during the culture of *Monascus* on adlay is less. The main objective of these studies was to increase *Monascus* pigment production by its culture on different types of adlay and to develop a continuous process for solid-state fermentation.

Adlay angkak had higher concentrations of red pigments than rice angkak when *Monascus* was cultured on cooked grains with 50% water content in 250 Erlenmeyer flasks. Maximum red pigment production on cooked whole grain adlay was obtained at a moisture content of 60 %. Adding sugar to adlay increased the pigment formation and fructose was better than glucose and sucrose. Adding a nitrogen source had more effect on the pigment production of adlay angkak than a carbon source. Adding histidine was superior to using monosodium glutamate (MSG) and sodium nitrate. Red pigment production was increased from 9.5 to 77 OD units per gram of dry matter when 2% histidine was added to adlay. Co-culture of *Monascus* with yeast only slightly increased the pigment yield.

Puffed adlay material was found to be a superior material for angkak production compared to whole grains. Optimization of the production process on 100 g puffed adlay showed that adding 4 g histidine, 20 g sugar and 200 g of water increased the pigment concentration 10 fold in fixed tray bioreactors. Oxygen transport limitation in a static bed normally limits angkak production to a layer of a few cm. Fine particles formed a bed which gave more problems with oxygen transport than whole grain particles. Mixing the bed can overcome such problems but cooked whole grain adlay was difficult to mix due to its high adhesivity. It was discovered that cooked puffed adlay at a moisture content of 60% gave high productivity yet low adhesitivity, and could be mixed easily. A rotating drum bioreactor was used to produce angkak on puffed adlay. In a continuously rotating culture at low speed, cells were damaged by shear. An intermittently rotating reactor gave poorer mixing but higher yield. The best condition was an intermittent speed with a low rotating speed and frequency of rotation.

Solid state culture on ground adlay showed severe transport limitations. The use of audible sound and ultrasound enhanced pigment formation in ground adlay. The penetration of the pigment into a bed exposed to audible sound vibration was limited to a thin layer of material near the surface, whereas at the optimum power and exposure times the use of ultrasound doubled the pigment yield.

Submerged culture on ground adlay was successfully performed in miniaturized stirred bioreactors. Increased agitation intensity improved yield, possibly indicating that oxygen transfer is limited in the miniature bioreactors.

The use of puffed grain during solid state culture and ground grain during submerged culture both have advantages for the production of angkak. Both of them can be applied in both batch and continuous processes. The particles of puffed grain make it possible to rotate the substrate during fermentation due to puffed adlay having a reduced stickiness. Overall, a production process based on the use of puffed grain in a solid state fermentation is better than one based on ground grain in a submerged culture because as the water demand of the process will be lower, there will be less waste generation and product stability will be higher. The addition of chemical supplements such as histidine, MSG and octanoic acid to a fermentation in a rotating system could further increase pigment formation.

DEDICATION

This thesis is dedicated to my parents, Chuan Maniyom and Sujitra Maniyom my sister, Tippawan Maniyom, my brother, Supachit Maniyom and also my wife and best friend, Patcharee Pattanagul who has supported and encouraged me throughout my period of research.

DECLARATION STATEMENT

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ACKNOWLEDGMENTS

This research was supported by a scholarship from MOST (The Ministry of Science and Technology, Thailand). In addition, there was some funding from Chiang Mai University. I would like to acknowledge my deep gratitude to my supervisor Professor Gerard H. Markx, Professor of Bioprocess Engineering, at Heriot-Watt University. He supported me in my research and advised me during my time at the university. I would also like to thank Professor Gerard H. Markx for inviting me to join his research group and use all the facilities in his laboratory. It is truly an honour to know him and his family.

I would like to thank Dr. Stephen Euston, School of Life Science, Heriot-Watt University for allowing me to use his measuring instruments. Thanks are also due to Aber Instruments Ltd., Aberystwyth, Wales for sponsoring me to work on the biomass monitor. Special thanks go to Eileen McEvoy, Vicky Goodfellow, Marian K. Millar, Ronald Miller, Curtis L Abbott, George R Smith, Cameron Smith, Rebecca Crawford and other staff members at the School of Engineering and Physical Science, and School of Life Science for their invaluable help and support. My gratitude also goes to the other members of my research group Ankita Singal, Manuel Garcia-Albornoz, Rama Yusvana, and Sneha Agarwal for their enthusiasm and friendship.

Additionally, I would like to thank Dr. Rory Mackenzie and his wife, Rosalyn Mackenzie, Miss Dorothy Nisbet, Kuck, Ploy and Thai student friends in the Christianity group, and also Scottish friends from the St Catherine's Argyle Church for their friendship and help in adjusting to life in Scotland. Finally, I would like to thank God for blessing me, my wife and family in Thailand.

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LIST OF ABBREVIATIONS AND NOMENCLATURE

C	Capacitance
cfu	Colony forming unit
cm	centimetres
CO_2	Carbon dioxide
C_p	Specific heat
^oC	Degree of Celsius
C^*	Saturated oxygen solubility
C_L	The actual dissolved oxygen concentration
Di	Impeller diameter
DO	Disolved oxygen
ε	Permittivity
F	Pumping rate
FDA	Food and Drug Administration
h	hours
g	grams
gds	grams dry solid
k_La	Volumetric oxygen transfer coefficient
L	litres
Hz	Hertz
kHz	kiloHertz
m	metres
M	Molar
MSG	Monosodium glutamate
O_2	Oxygen
OD	Optical density
MBCRs	Miniaturized bubble column reactors
MBRs	Minibioreactors
mg	milligrams
mL	millilitres
MHz	Megahertz
mJ	millijoules
min	minutes
mL	millilitres

mm	millimetres
mS	milliSiemens
MSBRs	Miniature stirred bioreactors
MTP	Microtitre plates
N	newtons
n	Rotation speed of impeller
ng	nanograms
PDA	Potato Dextrose Agar
PDMS	Polydimethylsiloxane
pF	picofarads
P/V	Power consumed per unit volume of liquid,
q_{O_2}	Specific uptake rate of oxygen
R or *r*	Cell radius
Rev	Revolution
Re	Reynolds number
rpm	Revolutions per minute
sec	seconds
SSF	Solid state fermentation
SMF	Submerged fermentation
SEM	Scanning Electron Microscope
$	Dollars
µg	micrograms
µm	micrometres
µ	Liquid viscosity
ρ	Liquid density
Vvm	Volume of air per total volume of bioreactor per minute
V	Volume of bioreactor
WHO	World Health Oganization
W	watts
X	Cell concentration
Yx/o_2	Yield coefficient on oxygen
ν	Impeller tip velocity

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Angkak or red yeast rice is a product resulting from rice fermentation using *Monascus spp*. *Monascus spp.* can produce coloured pigments which are yellow and orange and red (Rosenblitt *et al.*, 2000). Angkak has been used as a natural pigment in fish (surimi and other marine products), Chinese cheese, red wine, tomato ketchup and meat products (sausages and hams). The biggest angkak production is in China. In 2010, angkak production in China was 10,000 tons and it was valued at $150 million. Angkak products from China were supplied to Japan, the Republic of Korea, the United States, Mexico and other countries (Luo, 2012 and http://trade.e-to-china.com/trade-data/china-import-export-monascus-pigment.html_19Dec12). At present, there is a trend in angkak research to change from using rice as a raw material to other agricultural products such as corn, oats, wheat, cassava, palm kernels, groundnuts, tamarind seeds and jackfruit seeds. Adlay (Chinese pearl barley), a horticultural plant, is one of the most interesting products. It is used in traditional Chinese medicine as a nourishing food, due to its high nutritional value. In addition to this, it has alleged medicinal properties and has been used in the treatment of warts, chapped skin, rheumatism and neuralgia. It has also been alleged to have antitumour activity (Yang *et al.*, 2004). On top of this, when adlay is used as a substrate for angkak production little or no citrinin is produced (Chang, 2001 and Yang *et al.*, 2004). Citrinin is a mycotoxin which can be produced under certain conditions by *Monascus spp*. on rice angkak. The potential production of this mycotoxin by *Monascus* has caused the production and import of angkak to Europe and the USA as a food supplement to be prohibited.

The production of angkak on adlay has been studied very little. Pigment production can be divided into two processes: solid state and submerged culture. Traditionally, red yeast rice has been produced on a commercial scale in southern China, the Philippines, and Indonesia by solid state fermentation (SSF), mainly in tray bioreactors (Rosenblitt *et al.*, 2000). Submerged culture is more recent (Carvalho *et al.*, 2006), but is the main method used on an industrial scale for angkak production. Due to

the fact that the conversion of the solid material is slower, and the transport processes in the bed fermentation are limited to a layer of a few centrimetres in the tray bioreactors, as a result solid state fermentation (SSF) takes much longer and has higher labour costs. Because the low solubility of oxygen in water submerged culture also has oxygen transfer limitations, it is more easily controlled in submerged culture and the scale-up is more straightforward (Dominguez-Espinosa and Webb, 2003 and Rosenblitt *et al.*, 2000). Product concentrations obtained in SSF however are much higher than in submerged fermentation, potentially leading to reduced costs for product recovery and lower water and energy use. For SSF to be viable as an industrial process, further work is needed to improve the production process.

1.2 Aims

The aim of the research is to develop processes and find out the optimal conditions for pigment production by *Monascus* on adlay.

The objectives of this research are to:

- Quantify differences between adlay and rice as substrates for the culture of *Monascus*
- Study the effects of chemical and biological supplements on pigment production by *Monascus* when grown on adlay and to optimize pigment yield.
- Study mass transport processes during solid state culture by *Monascus* on adlay to develop mixed solid state cultures which allow the production of a homogenous product.
- Study mass transport limitation during culture of *Monascus* on ground grain.

To date, research on SSF culture of *Monascus spp.* has been limited because the culture time is long and takes more than three weeks. Also, conventional fermenters are too expensive and labour intensive for screening purposes. The use of miniature bioreactors will be explored as they would make it possible to run large numbers of reactors in parallel at relatively low cost. Miniature bioreactors also have high mass transfer capabilities, allowing rapidly metabolizing, high-cell density microbial cell cultivations to be supported, giving high productivity per unit volume (Betts and Baganz, 2006).

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

In this chapter, a description of angkak, its applications and limitations, and the problems associated with its production is given. The benefits of *Monascus* culture on adlay are also described. Furthermore, information is provided about *Monascus* and its culture - solid state culture and solid-liquid culture. Finally, information will be given about miniature bioreactors and the scaling up and down of fermenters.

2.2 Angkak

Angkak is a natural red colourant which is usually made by culturing *Monascus spp.* on cooked rice. For many centuries, people from China have produced angkak (red pigment) by growing *Monascus spp.* (red yeast rice) on rice. Common names for this fungal product are red yeast rice, red rice, angkak, red leaven, beni-koji (Japan), hung-chu, hong qu, zhitai (China), rotschimmelreis (Europe), red mould (U.S.A). It is the most globally used natural colouring agent (Carvalho *et al.*, 2005). In China, Indonesia, Japan, Korea, the Philippines, Taiwan, Thailand and the United States, it has been used as a food additive in products such as red soybean cheese, red rice wine, fruit flavoured yoghurt, meat products (sausages and hams), and marine products (surimi, fermented fish and fish paste), and as a dyestuff for textiles and leather (Panagou *et al*., 2003, Erdooğrul and Azirak, 2004, Dufossé *et al*., 2005 and Wang and Lin, 2007). Furthermore, the use of *Monascus* pigments has been suggested as a colourant for jellies, jam, ice cream, tomato ketchup and kamboko (Delgado-Vargos and Paredes-Lopez, 2003). More than 50 patents have been issued in Japan, the United States, France and Germany, concerning the use of angkak for food purposes (Dufossé *et al*., 2005).

The genus *Monascus* was first described in the West by van Tieghem in 1884. The genus *Monascus* belongs to the group of Ascomycetes and particularly to the family of *Monascaceae* (Carvalho *et al.* 2005 and Sabater-Vilar *et al*., 1999). *Monascus*

is characterised by the formation of ascospores which are of a spherical shape, 5 microns in diameter, or slightly ovoid (6×5 microns). The genus *Monascus* is divided into three major species: *M. pilosus*, *M. purpureus*, and *M. ruber*. *M. pilosus* and *M. purpureus* are more important for pigment production, while *M. ruber* is strongly associated with the decomposition of certain foods. Figure 2.1(a) shows some colonies in a Potato Dextose Agar (PDA) Petri dish, Figure 2.1(b) shows aerial mycelium and Figure 2.1(c) shows the liberation of spores.

All species can produce coloured pigments. The young part of the mycelium is often white in the early stages. However, this rapidly changes to a rich pink and subsequently to a distinctive yellow-orange colour. The production of yellow orange hyphae is thought to be a reflection of an increased acidity of the medium. A deep crimson colour is often found in the substrate when the culture ages (Erdogrul and Azirak, 2004, INPR, 2006 and Yongsmith, 1999).

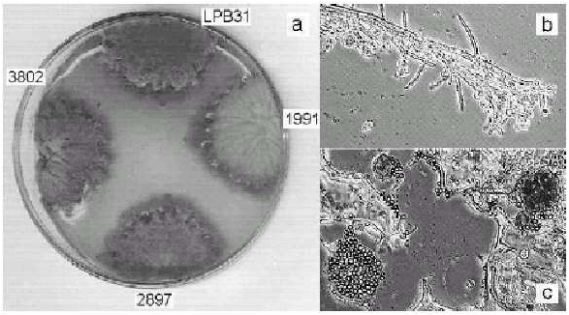

Figure 2.1 *Monascus* cultures of different strains after 8 days on Potato Dextrose Agar at 30-32°C (Source: Carvalho *et al.*, 2005)
(a) macroscopic view of four strains (*Monascus purpureus* NRRL 2897, *Monascus spp.* NRRL 1991, *Monascus purpureus* CCT 3802 and *Monascus purpureus* LPB 31)
and (b, c) microscopic view of LPB31, at a total magnification of 400 times.

Temperature, pH, humidity and oxygen concentration are important factors that influence the growth of microorganisms and pigmentation. All *Monascus* species are unable to grow anaerobically using glucose as a substrate, but can grow under oxygen limiting concentrations. Under these conditions, there is a higher production of ethanol and CO_2, but pigment production is lower. With higher aeration, ethanol production decreases whilst pigment production increases (Carvalho *et al.*, 2006). Apart from pigments, *Monascus spp*. can also produce enzymes, vitamins, flavour compounds,

polyunsaturated fatty acids, ketones, acetate and other compounds (Yang *et al*., 2004; Babitha *et al.*, 2007 and Yongsmith, 1999).

Six major types of coloured pigment can be distinguished in angkak which vary in colour from bright yellow (ankaflavin and monascin), orange (monascorubin and rubropunctatin), to deep red (monascorubramine and rubropunctamine) (Babita *et al.*, 2007, and Rosenblitt *et al*., 2000). The pigments are soluble in ethanol and slightly soluble in water. They can be found in concentrations of 10 – 100 mg g^{-1} dry wt matter (Krairak *et al.*, 2000 and Zheng *et al.*, 2009). All pigments are polyketides. *Monascus* pigments are not mutagenic but they can inhibit the mutagenicity of cooked meat and suppress tumour formation in mice which, of course, is important for the chemoprevention of cancer (Delgado-Vargos and Paredes-Lopez, 2003).

Figure 2.2 *Monascus* fungal products (Source: http://www.tianyibiote.com)

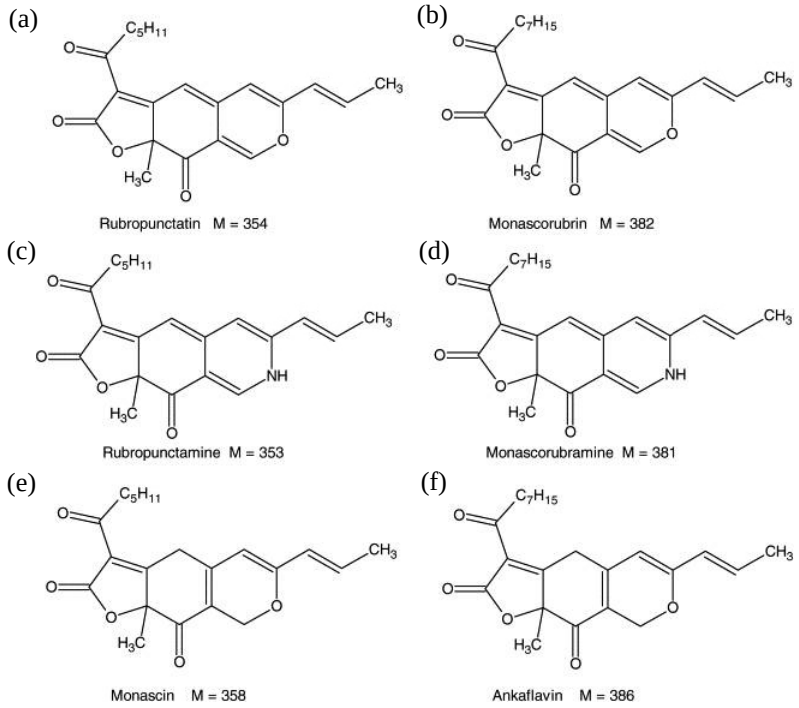

Figure 2.3 Pigments produced by *Monascus spp.* (Source: Dufossé *et al*., 2005).

(a) Rubropunctatin ($C_{21}H_{22}O_5$) (b) Monascorubrin ($C_{23}H_{26}O_5$)

(c) Rubropunctamine ($C_{21}H_{23}NO_4$) (d) Monascorubramine ($C_{23}H_{27}NO_4$)

(e) Monascin ($C_{21}H_{26}O_5$) (f) Ankaflavin ($C_{23}H_{30}O_5$)

Interest in red-yeast rice has recently increased, not only as a source of natural pigments for the food industries but also as a source of other secondary metabolites with cholesterol lowering properties. Mevinolin ($C_{24}H_{36}O_5$, also known as lovastatin, monacolin K, mevacor, 6-α-Methylcompatin and IUPAC 2-Methyl-1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-(tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl)ethyl]-1-naphthalenyl ester butanoic acid) is a secondary metabolite which can be found at concentrations of around 1 mg g^{-1} dry wt matter in angkak (Li *et al*., 2004, Praveen and Savitha, 2012, Yang *et al*., 2004 and sigmaaldrich.com). Mevinolin is biosynthetically derived from two polyketide chains joined through an ester linkage. One chain is the diketide 2-methylbutyrate and the other is a nonaketide that includes a distinctive conjugated hexahydronaphthalene ring system. Mevinolin is also produced by the fungi *Penicillium spp*. and *Aspergillus terreus* (Endo, 1979, Li *et al*., 2004, López *et al*., 2003). It is a member of the statin family of drugs and has the ability to reduce cholesterol levels in the circulatory blood system of animal models (such as rabbits and quail) and the human body. It inhibits 3-hydroxy-3-methyglutaryl coenzyme A reductase (HMG Co-A reductase), the rate limiting enzyme of cholesterol biosynthesis. Consumption results in a reduction in cholesterol levels. High cholesterol levels are a major risk factor for the development of coronary artery disease (Li *et al*., 1998, Li *et al*., 2004, Praveen and Savitha, 2012, Sayyad *et al*., 2007, Yang *et al*., 2004, Wang and Lin, 2007). Clear evidence exists that also shows that the use of Lovastatin has benefits for the treatment of strokes and can suppress tumour formation *in vivo* tumour by inhibiting the synthesis of non-sterol isoprenoid compounds. About 20 - 55% of reduction in site specific cancers (colorectal, breast, prostate, lung and pancreatic) was observed with the use of statin therapy. Furthermore, preclinical studies have reported that nanoparticle delivery of lovastatin may stimulate recovery of human bone fractures (Praveen and Savitha, 2012 and Sayyad *et al*., 2007). The United States Food and Drug Administration approved the use of mevinolin in August 1987 (Praveen and Savitha, 2012 and Sayyad *et al*., 2007).

Monacolin K can exist in two forms i.e. a beta-hydroxy acid form and lactone form (Figure 2.4). The hydroxyl form, the active drug, is hydrophilic as the lactone form is lipophilic (Praveen, and Savitha, 2012). The ratio of the acid form to the lactone form varies, depending on, the various *Monascus* strains used, the sample pH and other factors, such as the culture media. *Monascus* products containing higher ratios of the

acid form of Monacolin K should be regarded as a higher quality product (Ganrong *et al*., 2000). Furthermore, Monacolin J, L, X, M and its derivative forms were found from *Monascus* fermented rice. All types of monacolins have been found to be effective as hypocholesterolemic agents (Li *et al*., 2004).

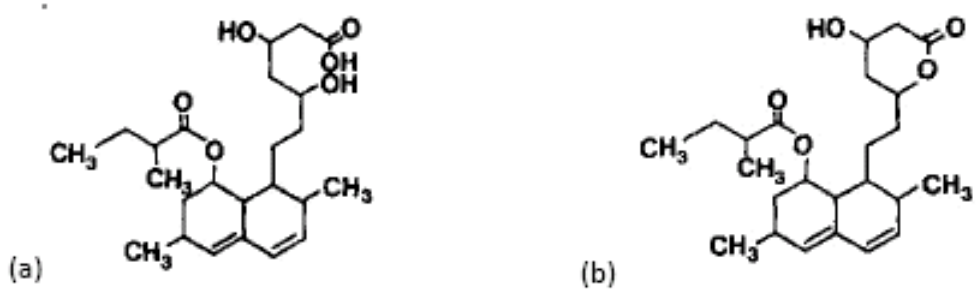

Figure 2.4 Chemical structure of mevinolin. (a) Acid form (b) Lactone form (Source : Friedrich *et al*., 1995, Li *et al*., 2004 and Praveen, and Savitha, 2012)

Citrinin was first isolated from *Penicillium citrinum* prior to World War II. Subsequently, it has been identified in over a dozen species of *Penicillium* and several species of *Aspergillus* (e.g. *Aspergillus terreus* and *Aspergillus niveus*), including certain strains of *Penicillium camemberti* (used to produce cheese) and *Aspergillus oryzae* (used to produce sake, miso and soy sauce) Citrinin has been reported to contaminate grains, food and feedstuffs such as wheat, oats, rye, corn, barley and rice (Sabater-Vilar *et al*., 1999; Xu *et al*., 2005 and Bennett and Klich, 2003). Citrinin ($C_{13}H_{14}O_5$, IUPAC (3R, 4S)-4,6-dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-3*H*-2-benzopyran-7-carboxylic acid), also known as monascidin A and hepato-nephrotoxin: mycotoxin, inhibits cholesterol and triglyceride synthesis in all animal species tested. This inhibition can cause damage to transport systems and/or interferences in energy metabolism (Blanc *et al.*, 1995, Bennett and Klich, 2003, Carvalho *et al.*, 2005, Ganrong *et al*., 2000 and Sabater-Vilar *et al*., 1999). Its acute toxicity varies from species to species. The 50% lethal dose is 35 mg/kg for mice, 67 mg/kg for rats, 57 mg/kg for ducks, 95 mg/kg for chickens and 134 mg/kg for rabbits (Bennett and Klich, 2003 and Wang *et al*., 2003). Most *Monascus* strains also have the potential to biosynthesize the mycotoxin citrinin. Angkak made from different materials (dioscorea, rice with or without added glucose, ethanol, fatty acids or acetate has been reported to contain citrinin (Blanc *et al.*, 1995, Hajjaj *et al.*, 2000, Lee *et al.*, 2006 and Lee *et al.*, 2007). This metabolite poses a serious problem for the use of angkak as a food additive and dietary supplement.

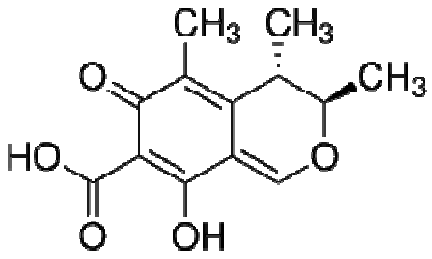

Figure 2.5 Chemical structure of citrinin

(Source : sigmaaldrich.com and Xu *et al.*, 2006)

Because the presence of citrinin remains a serious concern, the import of angkak into some countries has been restricted by agencies such as the FDA [Food and Drug Administration], the European Union, and the WHO [World Health Organization] (Delgado-Vargos and Paredes-Lopez, 2003). Although recent research has confirmed that red fermented rice posed no threat to health, most researchers agree that action should be taken to control the citrinin concentration (Chen and Xiaoqing, 2005). *Monascus* pigments are currently used in Japan, China and some countries in Southeast Asia, but their use is restricted in Europe and the USA. Much of the research on *Monascus* includes research on the reduction of citrinin content (Krairak *et al.*, 2000). In China and the European Economic Community, the citrinin level in red fermented rice is still under debate whilst a maximum level of citrinin allowed in red fermented rice in Japan has been authorized (Chen and Hu, 2005).

Citrinin levels in *Monascus* extracts at up to 1.8 µg g^{-1} extract are not mutagenic, as determined with a *Salmonella* microsome assay (*Salmonella* hepatocyte assay) with or without activation (Delgado-Vargos and Paredes-Lopez, 2003). The citrinin concentrations in commercial samples of angkak, however, have been shown to vary between 0.2 – 17.1 µg g^{-1} dry wt matter (Sabater-Vilar *et al.*, 1999). Some wild *Monascus spp.* can produce up to 500 µg g^{-1} citrinin (Zheng *et al.*, 2009). Many methods have been developed to reduce mycotoxin production in *Monascus*. This has included screening new *Monascus* strains and producing mutants with neutron bombardment, X-rays, ultraviolet light and chemicals, as well as modifying culture media and changing the growth conditions (Schmitt and Blanc, 2001 and Sabater-Vilar *et al.* 1999). For example, Schmitt and Blanc (2001) produced a mutant strain *Monascus spp.* (M12-69) from a wild strain from China after treatment with mutagenic agents

such as ultraviolet irradiation, ^{60}Coγ irradiation and dimethyl sulphate. It produced 0.13 ng g^{-1}citrinin and 1.94 mg g^{-1} monakolin K. Another way to reduce citrinin production can be to control the biosynthesis of the metabolites (Schmitt and Blanc, 2001). Unfortunately, it is difficult to increase the production of pigments and at the same time reduce the citrinin content, as part of the metabolic pathway is the same for citrinin and the pigments (see Figure 2.6). Adding fatty acids to the medium was effective in favouring the synthesis of pigments but the citrinin production remained unchanged. Changing the nitrogen source was more effective. When glutamic acid was replaced by other amino acids in a culture of *Monascus ruber* in glucose liquid medium the citrinin levels were significantly reduced. Histidine was found to be the most effective nitrogen source. It inhibits citrinin production because it promotes the production of peroxide which can destroy citrinin (Schmitt and Blanc, 2001).

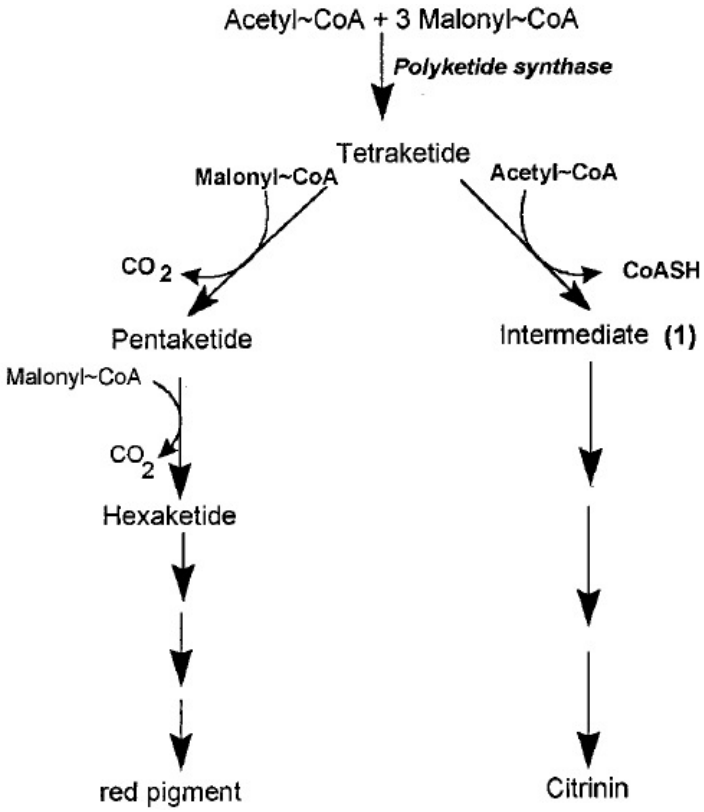

Figure 2.6 Biosynthesis of citrinin and red pigment in *Monascus ruber*
(Source: Hajjaj *et al.*, 1999)

2.3 Adlay

Adlay, scientifically named *Coix lachrymal-jobi L.*, is a grass crop from the Gramineae family (see Figure 2.7). It is called adlay in the Philippines, Job's tear in Australia, mayuen in China, sila in Fiji and Chinese pearl barley in Asia supermarkets. It is widely planted in many countries including Brazil, China, India, Japan, Philippines, Taiwan and Thailand (Hu *et al*., 2007, Shih *et al.*, 2004 and Yang *et al*., 2004). Adlay looks like sorghum and rice, has a height of 1 to 3 metres, and an erect stem with brace-roots. It has yellow, purple, white and black seeds. Adlay grain (Chinese pearl barley) is shaped like a pearl, is around 5 mm in diameter and weighs about 10 – 15 g / 100 seeds. It has a hard, shiny, dark brown to grey-black hull. The whole grain and its flour have long been used as animal feed, forage, and as ornamentation in necklaces and rosaries. It has also been used as nourishing food for humans and as a herb in traditional medicine, due to its high nutritional value and special biological and functional effects on the human body. It is used in soups and beverages and is popular in Chinese traditional medicine. Varieties of adlay (Chinese pearl barley) can be divided into three major types: *Coix lachrymal-jobi L.*var typica watt, *Coix lachrymal-jobi L.* var major mier and *Coix lachrymal-jobi L.* var mayuen. The first is used for decoration due it to having a very hard-shelled pseudocarp and strong differences in colour. The second variety is mainly planted and eaten in local villages because its shell is less hard. The last variety is widely harvested as a commercial cereal crop for sale in Asian supermarkets and has a soft-shell. It can be divided into two sub-groups: non-glutinous and glutinous. In the first group, the flour contains a lot of amylose whilst in the other group it contains a lot of amylopectin (97%) and only some amylose (3%) (Lakkham *et al.*, 2009 and Pornkitprasan, 1987).

Figure 2.7 Adlay plant and cooked adlay grains
(Source: http://th/wikipedia.org: *coix lacryma-jobi L.)*

Adlay has been used as a health food supplement and been reported to have medicinal properties. Its flour consists of 15% protein, 5.5% fat, 1.5% ash, and 0.5% fibre, 78% carbohydrate and 9% moisture. Moreover, it is rich in vitamins (vitamin A, vitamin B1 and B2), minerals (it is high in phosphorus), essential amino acids (glutamic, leucine, alanine, proline, valine, phenolalanine, isoleucine and arginine amino acids), free fatty acids (oleic and linoleic acids (84%) and palmitic and stearic acids (16%) and antioxidants (Lugli *et al*, 2002 and Pornkitprasan, 1987).

Several studies have found adlay (Chinese pearl barley) to be anti-allergic, and to have probiotic and hypolipidomic properties. According to the ancient Chinese medicine book *Pen-Tsao-Kang-Mu*, the seed of adlay was used in China for the treatment of warts, chapped skin, rheumatism and neuralgia and as an anti-inflammatory or antehelmintic agent. Furthermore, it has been widely used as a diuretic, stomachic, analgesic and antispasmodic agent. In addition, it has a stimulatory effect on the lungs, heart, striated muscles and smooth muscles and can dilate the pulmonary veins, improving the blood circulation of the lung. Coixenolide [$C_{38}H_{70}O_4$, 1-methyl-2-(cis-9-hexadecenoyloxy) propyl trans-11-octadecenoate], which was isolated from Chinese pearl barley seed, has anti-tumour activity towards Ehrlich ascites sarcoma in mice. It can prevent tumour formation and inhibit cancer cells such as those found in lung cancer and pancreatic cancer (Chang *et al*., 2003, Hu *et al*., 2007, Hung and Chang, 2003, Pornkitprasan, 1987, Shih *et al*., 2004 and Yang *et al*., 2004).

By growing *Monascus* on adlay one could potentially create a multi-functional food product - adlay angkak - with significant health benefits. A small number of publications have been published describing the formation of adlay angkak (Chang, 2001; Pattangul *et al.*, 2008) and analysis of angkak (Tseng *et al.*, 2004; Tseng *et al.*, 2006 and Yang *et al.*, 2004). *Monascus* adlay has been found not to contain citrinin at the detection limit of 1 $\mu g\ g^{-1}$ (Chang, 2001 and Yang *et al.*, 2004). In addition, the functional components of adlay are still present in the fermented adlay product along with those produced by fungus such as coixenolide, monacolin K, γ-aminobutyric acid, fatty acids and antioxidants. The monascal adlay product has been shown to have higher antioxidant activities and higher oxygen scavenging and chelating abilities than uninoculated adlay (Tseng *et al.*, 2004 and Tseng *et al.*, 2006).

2.4 The production of angkak by the fermentation of *Monascus*

The production of angkak has traditionally been done by a solid state fermentation. The classical Chinese method consists of inoculating steamed rice grains spread on large trays with a strain of *Monascus anka* and incubating it in an aerated and temperature-controlled room for 20 days (Dufossé *et al*., 2005). Production of *Monascus* pigment at a large scale has been conducted since the 1970's (Shin *et al.*, 1998). Commercial production nowadays is dominated by submerged culture. Red yeast rice is still produced on a commercial scale by solid substrate fermentation (SSF), mainly in tray bioreactors, in southern China, the Philippines and Indonesia (Rosenblitt *et al.*, 2000 and Carvalho *et al.*, 2006). During solid state culture, pigments are released at high concentrations into the grains, and are therefore easily extracted. Submerged fermentation is typically done in stirred tanks and bubble column fermenters (Kim *et al.*, 2002, Wang and Lin, 2007 and Wu *et al.*, 2000). During submerged cultivation, the pigments normally remain in mycelium due to their low solubility in the (acidic) medium (Jůzlová *et al.*, 1996). However, the pigments can react with amino group-containing compounds such as proteins, amino acids, amino sugar, amino alcohols, chitosan and nucleic acids in the liquid medium to form water-soluble pigments (Delgado-Vargos and Paredes-Lopez, 2003). Spores produced during solid state fermentation are more robust and more virulent than spores produced during submerged fermentation.

Figure 2.8 An experimental fermenter growing *Monascus purpureus* in the laboratory (Source: Moore and Chiu, 2001)

2.5 Influence of different factors on the production of angkak

Many factors affect biomass and pigment production during the culture of *Monascus*. They can be divided into parameters associated with the composition of the medium and the atmosphere, and process conditions.

Composition of the medium/substrate

1). Carbon source

Carbon sources that support growth are usually sugars (e.g. D-glucose, D-fructose and sucrose) which are rapidly taken up. Polysaccharides, amino acids, lipids, organic acids, alcohols and hydrocarbons are also used (Papagianni, 2004).

The selection of the solid substrate is critical for the success of a solid state fermentation. Substrates for solid state fermentations are polymeric in nature and are insoluble in water. They have to be converted to a soluble form before they can be taken up by the microbial cells and are inaccessible in the initial stages of growth. Most substrates used as solid substrates are able to support growth with or without fortification with additional nutrients.

Normally, the fermentation rate is controlled by the rate at which the moist solid is converted and taken up by the microorganisms. The rate of uptake will be influenced by various physical and chemical factors such as the shape and size of the solid particles, porosity, fibrousness, surface to mass ratio, crystallinity, amorphism, stickiness, diffusivity within the solid, hydrophobic or hydrophilic nature of the substrate etc. Pre-treatment can increase the rate of uptake. Pre-treatment is done by mechanical or chemical methods such as steaming, soaking, crushing, pearling, cracking, chopping, shredding, grinding, or by treatment with alkali or sodium chloride (Lonsane *et al*., 1985 and Velmurugan *et al.*, 2011).

Traditionally rice has been used for the cultivation of *Monascus* (forming angkak or red rice) although other media such as cassava, corn, dioscorea, oat, rice bran, soybean, wheat grain and wheat bran have also been used (Carvalho *et al.*, 2006, Jůzlová *et al.*, 1996, Lee *et al.*, 2006 and Lee *et al.*, 2008). The main reasons to change to new substrates are to increase pigment, or reduce costs. Carvalho *et al.* (2007)

compared broken rice, wheat, corn, soy, soy bran, textured soy protein (TSP), cassava starch, cassava flour, cassava bagasse and potatoes for the solid state culture of *Monascus*. Rice and cassava flour were superior for pigment production. Cassava bagasse was cheap but gave a low product yield due it containing only 66% starch. Corn has been used as a substrate for angkak production because it is cheaper than rice and is not consumed as a main dish in China (Palo *et al*., 1960). Jackfruit seed powder gave higher pigment yields compared to rice bran, wheat bran, coconut oil cake, sesame oil cake, palm kernel cake, groundnut oil cake, cassava powder, spent brewing grain and tamarind seed powder (Babitha *et al.*, 2007). Porosity of the material can affect pigment production. Lin and lizuka (1982) compared various kinds of substrate and found that steamed bread (mantou), which has a very high porosity, gave the best pigment yield.

In submerged culture, *Monascus* grows quite well on starch, maltose, fructose, raffinose, dextrin, galactose, and glucose, respectively (Omamor *et al.*, 2008). High production rates of pigments can be achieved using starch, glucose, maltose and fructose, (Lee *et al*., 2001). A glucose concentration within a range of 4 – 10 % gives high monacolin K production rates (Lin *et al.*, 1991 and Wang and Lin, 2007), but a glucose concentration of more than 50 g/l has been shown to inhibit cell growth and pigment synthesis as well as causing significant ethanol production (Jůzlová *et al.*, 1996). It has been recommended that the glucose level should be maintained below 20 g/l (Krairak *et al.*, 2000).

The use of an ethanol solution as a substrate has been shown to have a negative effect on the growth of fungi but it increased the production of red pigment. Adding ethanol is a very good carbon source for pigment formation. However, ethanol concentrations of over 2% inhibited both growth and pigment production (Wang and Lin, 2007).

Tapioca starch has been successfully used in the submerged culture of *Monascus*. *Monascus* growing in submerged culture on 50 g/l tapioca starch as carbon substrate had 8 g/l biomass, 31 OD units of red pigment and 26.5 OD units of yellow pigment (Lee *et al.*, 1995). When fructose, ethanol, galactose, glucose, lactose, maltose, sucrose and soluble tapioca starch were compared, soluble tapioca starch proved to be better than other carbon sources in terms of both growth and pigment production (Lee *et al.*, 2001).

Dominguez-Espinosa and Webb (2003) showed that medium viscosity played a role when increasing the substrate concentration during submerged culture. *Monascus* grew on wheat flour in shaken flasks. The fungal *Monascus* biomass yield increased when the flour concentration was increased from 2 to 7 %, but at 8 to 9% flour the biomass production became lower again. The highest pigment concentration was achieved at 5% (w/v) of flour. The initial viscosity of the medium was found to affect the morphology of the fungus. Pellets were formed when the initial viscosity was lower than 100 cp. Pellet sizes were diminished and mycelia were more dispersed as the initial broth thickness increased. When the concentration of flour was higher than 10% w/v, aeration of the medium was poor and consequently pigment production was minimal (Dominguez-Espinosa and Webb, 2003).

Adding fatty acids as supplements has been shown to be effective in favouring the synthesis of the pigment but citrinin production remained unchanged (Hajjaj *et al*., 2000 and Dufossé *et al*., 2005). Hajjaj *et al.,* 2000 reported that octanoic acid was the best fatty acid. It increased pigment production more than hexanoic acid, decanoic acid, dodecanoic acid, myristic acid, stearic acid and oleic acid did.

By-products of the food industry such as sugar cane molasses, cheese whey, corn steep liquor, industrial soybean residue, and grape waste of the wine industry have also been reported to be good substrates for red pigment production (Hamano and Kilikian, 2006 and Silveira *et al.*, 2007).

2). Moisture content

The moisture level of the substrate is one of the key factors influencing the outcome of a solid state fermentation. Generally, oxygen diffusion in air is much easier than in water. Too high water content level results in lower rates of oxygen diffusion and enhanced formation of aerial mycelium, reduction in gas volume and decreased gas exchange whereas too low a moisture level leads to sub-optimal growth, a lower degree of swelling and high water tension. The initial moisture content is usually in the range of 30 - 75% of water content but it changes during fermentation due to evaporation and metabolic activities (Lonsane *et al*., 1985). Initial moisture content in wheat bran is between 50 - 60% but in sugarcane bagasse it is around 70%. Pigment yield varies in different materials from 50 - 90% (Carvalho *et al.*, 2007). Babitha *et al.* (2007) reported

that an initial substrate moisture level of less than 40% in *Monascus* cultures usually gave less pigment, and one of 50 – 56% generally gave the highest amount of pigment. A moisture content of more than 70% usually resulted in the agglomeration of the substrate, subsequently restricting the supply of oxygen for the growth of the microorganism. At moisture levels over 80% the culture medium becomes too wet with free water and agglutinated (Carvalho *et al.*, 2007). The highest pigment yield in cassava bagasse however was 70% of moisture. This is due to it having a high fibre content (Carvalho *et al.*, 2007).

3). Nitrogen source

The nitrogen source seems to be more important than the carbon source. Nitrogen may be supplied as ammonia, nitrate and organic compounds such as amino acids and proteins. Beet or cane molasses, corn steep liquor, whey powder, soy flour, yeast extract and others are used as industrial raw materials rich in nitrogen (Papagianni, 2004). Low concentrations of nitrogen in the medium had a detrimental effect on both growth and colourant production (Dominguez-Espinosa and Webb, 2003). Omission of nitrogen in the medium greatly affected fungal growth and metabolite production. Without nitrogen the mycelium has a low metabolic activity and the hyphae appear partially empty and broken at the tip. At low nitrogen levels, fungal pellets are small, very light and fluffy, and the fungus has an increased tendency to form clumps (Papagianni, 2004). In general, the growth of cells is favored at a C/N ratio value close to 50 g g^{-1} whilst the production of pigments is favoured in the region of 7 – 9 g g^{-1} (Dufossé *et al*., 2005 and Wang and Lin, 2007). Furthermore, nutrient formulas can also be modified to increase pigment production and reduce citrinin content (Dufossé *et al*., 2005).

The use of yeast extract increased mycelium formation and reduced pigment formation (Jůzlová *et al.*, 1996). Furthermore, the use of yeast extract sucrose medium (YES medium) in 23 *Monascus* type cultures was found to result in high citrinin levels from 65 to 480 mg/l in all these strains (Wang *et al*., 2005). The addition of sodium nitrate has been shown to support sporulation, limit growth and lead to intermediate pigment yields (Jůzlová *et al.*, 1996). It also represses aflatoxin formation (Wang and Lin, 2007). The addition of ammonium chloride increased pigment yields because it can decrease pH dramatically, due to the impaired pigment-amine interactions to red

pigment (Jůzlová *et al.*, 1996). When various agro-industrial residues such as carboxymethylcellulose (CMC), cheese whey powder, soy bran and grape waste were augmented with peptone, NH_4Cl and/or soy bran, it was found that peptone influences the production of β-glucosidase and red pigment more than other nitrogen sources (Daroit *et al*., 2007).

Monosodium glutamate (MSG) has been consistently found to be a good nitrogen source. Monosodium glutamate was found to be the most favourable nitrogen source for the formation of pigments in a culture in MOPS-buffer (3-[N-morpholino]-propanesulphonic acid buffer) whereas ammonium glutamate gave superior pigment yield in unbuffered cultures (Daroit *et al*., 2007, Dufossé *et al*., 2005, Jůzlová *et al.*, 1996, Wang *et al*., 2005 and Wang and Lin, 2007). Lee *et al.* 2001 compared ammonium chloride, ammonium nitrate, ammonium sulphate, monosodium glutamate (MSG) and peptone, soybean meal, sodium nitrate and yeast extract in submerged fermentation. The use of ammonium nitrate, sodium nitrate and monosodium glutamate (MSG) led to good pigment production but produced less biomass. Mukherjee and Singh, 2011 reported that monosodium glutamate (MSG) was the best nitrogen source in submerged fermentation when compared with ammonium chloride, ammonium nitrate, ammonium sulphate, potassium nitrate, sodium nitrate and peptone. Vidyalakshmi *et al.*, 2009 reported that when ammonium nitrate, monosodium glutamate (MSG), potassium nitrate, sodium nitrate, peptone and yeast extract were added to a submerged fermentation on rice, monosodium glutamate (MSG) was the best nitrogen source. Lin and Demain (1991) reported that 1.26% monosodium glutamate (MSG) was optimal for growth and pigment production when compared to a maltose solution with ammonium chloride and ammonium nitrate.

The addition of amino acids can in some cases increase production, and in some reduce it. Carels and Shepherd (1978) reported that the addition of amino acids did not increase mycelium formation but increased the amount of conidation. Pigment production was actually reduced. This was thought to be due to the fact that the addition of the amino acids inhibited nitrate and ammonia uptake. On the other hand, adding amino acids can stimulate the extracellular accumulation of pigment and affect citrinin production when *Monascus ruber* was cultivated in a liquid medium containing glucose and various amino acids (Dufossé *et al*., 2005 Jung *et al*., 2003 and Schmitt and Blanc, 2001). The addition of histidine can inhibit the production of citrinin (Dufossé *et al*.,

2005 and Schmitt and Blanc, 2001). A comparison between monosodium glutamate (MSG), soyapeptone and histidine indicated that the best results in pigment production were obtained using histidine in glucose and ethanol as substrate (Dufossé *et al*., 2005, Jung *et al*., 2003 and Schmitt and Blanc, 2001). In addition, it was found that L-malate, leucine, valine, lysine, methionine and succinate had strong negative effects on the formation of red pigments (Jung *et al*., 2003 and Jůzlová *et al.*, 1996).

4). Trace elements

Trace elements are essential nutrients which are required at low concentrations to support growth and the production pigment and other secondary metabolites. Zinc, manganese, copper, iron, heavy metals and alkaline metals can have an effect on fungal morphology. The addition of zinc and iron has a positive effect and is probably related to the diversion of carbon between biomass and citric acid. Manganese is involved in protein synthesis and many cellular processes such as cell wall synthesis and sporulation (Papagianni, 2004). Jůzlová *et al.*, (1996) and Wang and Lin, (2007) reported that when zinc sulphate is added, the growth of *Monascus spp.* is inhibited but the pigment production is promoted. Dominguez-Espinosa and Webb, (2003) reported that biomass and pigment by *Monascus* production were increased up to two times by the addition of zinc sulphate at a concentration of 0.1 g/l but ammonium chloride at 2 g/l had a negative effect on the pigment formation (Dominguez-Espinosa and Webb, 2003).

5). pH

Fungi can grow over a wide range of pH. Most of them tolerate a pH range from 4.0 to 9.0 but grow and sporulate maximally near neutral pH. The composition of the medium can affect the initial pH and how much it changes during fungal growth (Papagianni, 2004). The pH is a critical factor but control of pH during fermentation is not usually attempted during solid state fermentation. The initial pH in a solid state fermentation is usually in the range 4.5 – 5.0 (Lonsane *et al*., 1985). *Monascus* growth has been observed in a wide range of pH, from 2.5 to 8.0, with the ideal range being from 4.0 to 7.0 (Carvalho *et al.*, 2005). At a very low pH of 2 and 2.5, there was no fungal growth. The maximum growth was attained at pH 4. At higher pH values there was a decrease in the growth, even though the pigment yield gradually increased from

pH 4.5 to 7.5 (Babitha *et al.*, 2007). During submerged fermentation the pH of the medium is especially important in regulating the formation of conidia and pigment production. The pH change during cultivation depends on the nitrogen source and to a lesser extent, on the carbon source. pH affects the uptake of certain medium components, as well as its possible control of certain metabolic pathways which ultimately determine the availability of intermediates for conidiation and pigment. The optimal initial pH value must also be selected with respect to the carbon and nitrogen sources used. Lowering the pH inhibits the formation of conidia and increases pigment production; also the effect of the amino acid addition is totally annulled (Carels and Shepherd, 1978 and Jůzlová *et al.*, 1996). In submerged culture, *Monascus* favoured pigment production in pH range from 5.5 to 8.5. When the pH range was higher than 8.5 and lower than 5.5, the pigment production decreased noticeably (Lee *et al.*, 2001 and Mukherjee and Singh, 2011).

6.) Composition of the atmosphere

Oxygen and carbon dioxide levels strongly affect *Monascus* pigment production during solid state fermentations. Partial pressures for oxygen and carbon dioxide of 0.5 and 0.02 atm respectively have been recommended for breeding *Monascus spp.* (Jůzlová *et al.*, 1996). When the carbon dioxide level is maintained at 0.02 atm pigment production during solid state culture was doubled when oxygen levels were increased from 0.01 atm to 0.50 atm. On the other hand, increasing carbon dioxide levels decreased pigment production when oxygen levels were kept constant. High carbon dioxide at 1 atm completely inhibited pigment production and decreased fungal growth (Jůzlová *et al.*, 1996).

7). Co-culture with other microorganisms

Co-culture of two fungi during solid state fermentation can enhance enzyme formation and other products. The co-culture of *Trichoderma reesei* mutants with *Aspergillus spp.* increased cellulose production by 50%. Co-culture of *Aspergillus ellipticus* and *Aspergillus fumigatus* resulted in improved hydrolytic and β–glucosidase activity (Bhargav *et al.*, 2008). When *Monascus* was co-cultured with either *Saccharomyces cerevisiae* or *Aspergillus oryzae* in a solid sucrose medium, there were significant morphological changes in *Monascus*. Cell mass doubled and the pigment

yield increased 30-40 times compared to monocultures of *Monascus* (Hölker *et al.*, 2004, Shin *et al.*, 1998 and Wang and Lin, 2007).

8). Temperature

Temperature affects spore germination, growth, product formation and sporulation. Fungal growth is possible from 15-18°C (minimum) to around 45°C (maximum), with pigment production greatly varying with different species and cultivation conditions. The optimal temperature range for *Monascus* growth is typically 28-32°C, but can vary, depending on the strain, between 25°C and 37°C (Carvalho *et al.*, 2005 and Lonsane *et al*., 1985). For example, Babitha *et al.* (2007) studied the effect of temperature on pigment production by *Monascus* on jackfruit seed powder. Flasks were incubated at various temperatures. An optimum temperature was obtained between 30°C and 37°C for different isolates of *Monascus spp.*; maximum yellow pigment production was obtained at 30°C; pigment production decreased drastically beyond 40^{o}C (Babitha *et al.*, 2007 and Velmurugan *et al.*, 2011). The optimal temperature in submerged cultivation for individual *Monascus* strain varies from 25°C to 37°C. Nevertheless, the most frequently cited temperature is 30°C. A shift in temperature from 30°C to 23°C has been shown to increase lovastatin production by nearly 20 times (2.25 mg/g dry koji) compared to cultivation at a constant temperature of 30°C over 14 days (Jůzlová *et al.*, 1996 and Tsukahara, *et al.*, 2009).

High viscosities in submerged fermentations (SmF) can lead to heterogeneity inside the fermenter, as well as poor oxygen transfer and low pigment yield. Broth viscosity can be reduced by slowing down the fungal growth rate by culturing at a low temperature (25°C). As a result, the pigment yield at 25°C was 10 times greater than at 30°C (Ahn *et al.*, 2006 and Wang and Lin 2007).

9). Light

Light can cause the *Monascus* pigment concentration to decrease and in time to be completely destroyed. Cultivation of *Monascus* in total darkness is recommended (Jůzlová *et al.*, 1996). *Monascus spp*. gave maximum pigment yield in darkness and minimum pigment yield in white light (Feng *et al.*, 2012). The effects of other lights are different. The primary red pigments (monascorubramine and rubropunctamine) are less

photostable than yellow pigments. For example, after 170 h, pigment concentrations in cultures without light-shielding were 81%, 43%, and 38% lower for primary red, orange, and yellow pigments, respectively (Babitha *et al.*, 2008). Incubation in total darkness increased red pigment production from 14.5 to 22 OD units/ gram dry substrate (Babitha *et al.*, 2008). Miyake *et al.* (2005) reported that both red and blue lights affect development in *Monascus,* influencing mycelium and spore formation and the production of secondary metabolites. Jůzlová *et al.* (1996) on the other hand found that pigment formation during submerged culture is independent of visible light. When they irradiated *Monascus* cultures with light of various wavelengths they found that blue light, red light and infrared light did not affect pigment production

10). Aeration

Aeration of the fermentation mass in most laboratory scales and large-scale fermenters is achieved by forcing in sterile air under pressure. Aeration rates are governed by the nature of microorganism used, the amount of metabolic heat to be dissipated from the mass, the degree of air space available in the substrate, the oxygen requirement for the synthesis of the product and the rate at which carbon dioxide and other volatile metabolites need to be removed (Lonsane *et al.*, 1985).

Oxygen is an important substrate for fungal growth. Fungi can grow over a very wide range of oxygen concentrations but most fungi do require molecular oxygen to grow. Even interruption of the aeration in submerged culture for a short time can affect production. For example, it has been found that the interruption of the air supply for up to 20 minutes in an *Aspergillus niger* fermentation did not reduce the viability of the cells, but it resulted in a complete loss of the ability to produce citric acid. Carbon dioxide is an inhibitor of fungal growth, and not removing it quickly can reduce productivity. Inlet gas concentrations of more than 3% carbon dioxide in *Aspergillus niger* cultures have been shown to decrease biomass levels, substrate consumption, and citric acid concentration and also induce morphological changes (Papagianni, 2004).

11). Agitation

In solid state fermentation, agitation is uncommon. Agitation of the fermenting mass does however have advantages. It provides homogeneity throughout the

fermentation period, promotes growth on the substrate particles, may prevent aggregate formation and exposes individual substrate particles to the atmosphere (Lonsane *et al*., 1985). Mixing also helps to ensure effective heat and mass transfer (Lonsane *et al*., 1985). Agitation of the substrate, unfortunately, also causes high shear forces to be exerted which can damage the mycelia.

In submerged fermentations, agitation is nearly always used. Agitation has multiple functions. Agitation is important to achieve good mixing which helps in keeping substrate particles and cells suspended and the medium homogenous. It helps in enhancing mass and heat transfer. However, a major role of agitation in submerged fermentation is to ensure sufficient oxygen transfer throughout the vessel. Unfortunately agitation can create shear forces which can affect microorganisms in many ways, for example by damaging cell structures and causing morphological changes as well as variations in growth rate and production formation (Bhargav *et al.*, 2008). To prevent damage to microorganisms, the impeller speed or power input may have to be restricted (Papagianni, 2004). Different morphologies may exist at different agitation rates. At higher stirrer speeds, small, compact pellets may dominate, while a mixture of free filaments and loose pellets may exist at lower speeds (Papagianni, 2004). Different reactor types may also give different morphologies because the intensity of agitation is different. For example, Gerlach *et al.* (1998) found that during a xylanase fermentation of *Aspergillus awamori* large, loose, hairy pellets were formed in pneumatically mixed reactors (airlift tower loop), whilst small and compact pellets were formed in shaken flasks and stirred tank reactors.

2.6 The culture of filamentous fungi

The culture of *Monascus* is essentially the same as for other filamentous fungi, and it is therefore useful to look at how filamentous fungi are cultured in general. Filamentous fungi are used extensively to make a large variety of products commercially, in particular food, enzymes and small organic acids. The global value of enzymes and metabolites from filamentous fermentations is in the region of US$ one billion (Moore and Chiu, 2001).

Growth of filamentous fungi is either by solid state fermentation (SSF) or submerged fermentation (SmF). Solid state fermentation is a fermentation process which is carried out on a solid matrix in the absence or near absence of free water. In

contrast, during submerged culture the microorganism and substrate are dispersed in free water (Singhania *et al.*, 2009). The media used in solid state fermentation contains less water, and a gas phase exists between the particles (Durand, 2003). The substrate must possess enough moisture to support growth and metabolism of the microorganism. The water content of the mash in solid state culture is typically between 40% - 80% (Shuler and Kargi, 2002), whereas the water content of a typical submerged fermentation is more than 95%. Approximately 90% of all enzymes and secondary metabolites in industry are produced by submerged fermentation (Hölker *et al.*, 2004). The production of enzyme and organic acids are the most important applications of solid state fermentation. The advantage of solid state fermentation over submerged culture is the relatively low energy requirement of the process, the direct transfer of oxygen to the microorganism at the surface and the high yields of secondary metabolites (Barrios-González *et al.*, 2005). Generally, the yield of product per litre of extract obtained with solid state culture is 10 times more than with a submerged technique. If the same strain and identical substrate is used, the yield per gram of carbon substrate is about three times higher in solid state culture than in submerged culture (Lonsane *et al*., 1985). Furthermore, SSF processes have many advantages over the submerged fermentation cultures such as lower water demand, higher end-concentration of products, higher product stability, high productivity per reactor volume, lower demand for energy for heating, less waste generation, low cost of equipment involved, there being no need for anti-foaming chemicals and ease of aeration due to the cultivated fungi growing on the solid surface (Bhargav *et al.*, 2008 and Hölker *et al.*, 2004). The cost-factor for the production is in favour of SSF over SmF (Hölker *et al.*, 2004). It has been shown for a plant producing 9, 30 and 150 m^3 enzyme, respectively, that the total capital investment needed is 28, 23 and 18% of the total costs of production for solid state culture whilst it is 61, 52 and 58% for submerged culture (Lonsane *et al*., 1985). The cost of cellulase production with solid state fermentation is at US$ 0.2 kg^{-1} whereas with a stirred tank reactor it is up to US$ 20 kg^{-1} (Hölker *et al.*, 2004).

Yet solid state fermentation also has many problems associated with it. Solid state fermentation has high labour costs. Transport issues within the substrate bed cause the formation of gradient in nutrients, oxygen, pH and temperature. Maintaining optimum values of these factors on a large scale is very difficult. This results in long culture times, increasing the threat of contamination, and difficulties in scaling up. Only a few SSF processes have been successfully used in a commercial context (Hölker *et*

al., 2004, Viccini *et al.*, 2001 and Wang and Lin 2007). In many publications, solid state fermentation has been shown to give a better yield than submerged fermentation when secondary metabolites by various microorganisms were compared in solid state fermentation and submerged fermentation (Hölker *et al.*, 2004). No final decision on angkak has been made as to whether solid state fermentation or submerged fermentation is better.

2.7 Bioreactors for the culture of filamentous fungi

Typical bioreactors used for the culture of filamentous fungi are shown in Figure 2.9. Bioreactors used for the culture of filamentous fungi can be subdivided on the basis of whether they are used for solid state fermentation (SSF) or submerged fermentation (SmF). Most industrial microbial processes are aerobic, and high oxygen transfer rates are often needed. High oxygen transfer rates can be achieved by choosing a suitable type and design. Operation may be with or without forced aeration and agitation (Couto and Sanromán, 2006, Garcia-Ochoa *et al.*, 2009 and Pandey, 1991).

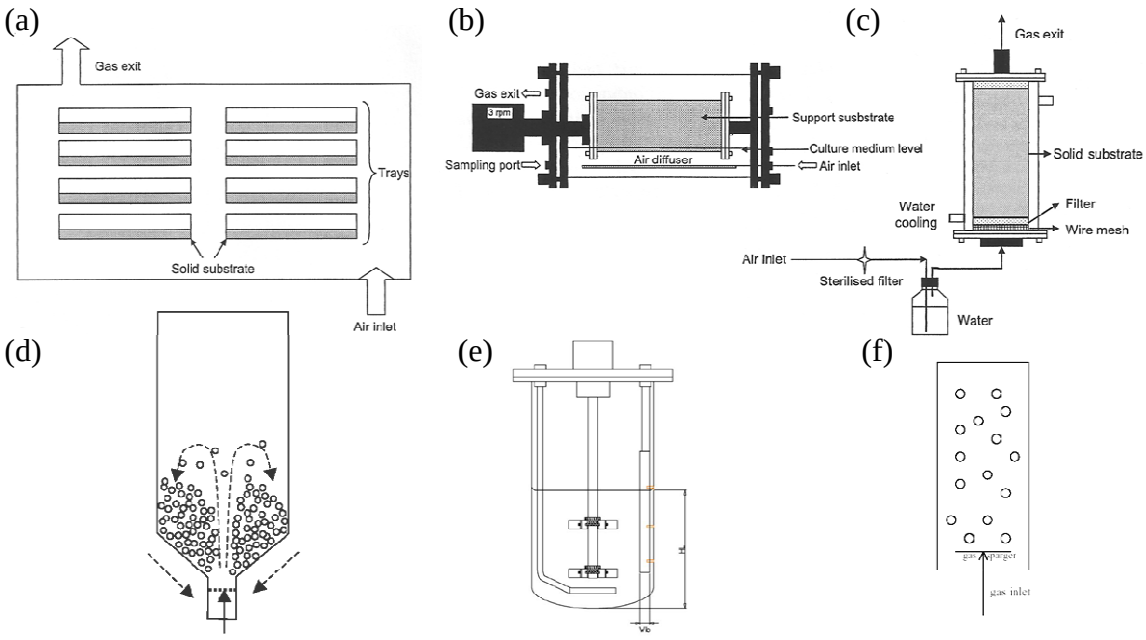

Figure 2.9 Types of fermenter used for the culture of filamentous fungi

a) Tray fermenter (b) Horizontal drum fermenter (c) Packed-bed fermenter

d) Fluidized bed fermenter (e) Stirrer tank fermenter (f) Bubble column fermenter

(Source: Couto and Sanromán, 2006, Mitchell *et al.*, 2006, Mohamed *et al.*, 2009 and Fu *et al.*, 2003)

Fermenters for solid state culture

Solid state fermenters can be divided into subgroups based on the aeration and agitation strategies. Classical bioreactors with these groups include tray bioreactors, packed bed bioreactors, rotating drum bioreactors, and various continuously or intermittently mixed bioreactors with forced aeration (Moore and Chiu, 2001).

1). Tray fermenters

Tray fermenters are the simplest type of fermenters. Tray fermenters have an unmixed bed and operate without forced aeration. The trays may be wooden, metallic (stainless steel or iron) or plastic (Pandey, 1991). Tray fermenters need a large operational area and are labour intensive. The design does not lend itself easily to mechanical handling. Scale up is done simply by increasing the number of trays (Bhargav *et al.*, 2008). Tray fermenters have been extensively used in industry, mainly in Asian countries (Durand *et al.*, 2003), even though it is an unattractive design for large-scale production (Pandey, 1991). Usually, substrate is spread on each flat tray forming a thin layer and trays are arranged one above the other with a suitable gap between the trays. The substrate forms a layer of about 1.5 or 2.0 cm in thickness (Couto and Sanromán, 2005). The fermentation is carried out in a chamber where a controlled humid atmosphere is created. Temperature of the fermenting substrate is controlled by circulating warm or cool air as necessary. Tray fermentation has been used for 1000's of years for the production of Koji in Japan and South East Asia, Miso in Japan and China, Tempe in Indonesia (Couto and Sanromán, 2006 and Pandey, 1991). Bicon developed and patented a new bioreactor, the PlaFractor^{TM} which was an automated tray culture process (Suryanarayan, *et al.*, 2003). All the operations of solid matrix fermentation, i.e. sterilization, cooling, inoculation, fementation, product recovery and post-fermentation sterilization are all done in the same bioreactor. The production takes place in computer controlled bioreactors using complex fermentation control algorithms.

2). Packed-bed fermenters

Packed-bed fermenters are often made from glass or plastic columns with lids at both ends. Packed bed fermenters have an unmixed bed through which air is forced. Substrate is retained on a perforated base in the column and humidified air is continuously forced through the bed of substrate. Some may be fitted with a jacket for circulation of water to control the temperature during fermentation. They have been used for the production of enzymes, aroma compounds, antibiotics and organic acids such as penicillin, citric acid and glucoamylase (Pandey, 1991). Packed bed column fermenters have been used in commercial koji production but their configuration makes it difficult to control the uniformity of the product and heat removal, and gives problems with scale up (Couto and Sanromán, 2006 and Pandey, 1991), and their use has been mainly confined to the laboratory.

3). Fluidized-bed fermenters

In fluidized bed bioreactors, the solid substrate is fluidized by an upward airflow. The constant agitation of the substrate particles by the airflow stops them from adhering to each other and aggregating. The bioreactor has to be high enough for bed expansion. Widening of the column near the top allows disengagement of solids from the gas stream. Although the mass and heat transfer, aeration and mixing of the substrate are increased, the cells may be damaged by the shear force of the air, and this may affect the yield of the final product (Bhargav *et al.*, 2008 and Couto and Sanromán, 2006). Furthermore, large agglomerates will form if sticky particles are used and these agglomerates will not fluidize (Mitchell *et al.*, 2006).

4). Horizontal drum fermenters

Drum fermenters consist of a horizontal drum-shaped reactor equipped with a rotating device, and they usually need an air inlet (Pandey, 1991). They have mainly been used in laboratory studies for Koji fermentation and enzyme production (Pandey, 1991). In drum bioreactors, the solid substrate is mixed by the rotating action of the vessels. The mixing of the substrate makes the substrate uniform and also solves heat transfer and mass problems (Bhargav *et al.*, 2008). Growth of the microorganisms in this type of bioreactor is generally considered to be better and more uniform than in tray

fermenters. At the same time, heat generation and aggregation of substrate particles into balls caused by the mixing can affect the yield. Process conditions have to be optimised to allow adequate aeration and mixing of the substrate, limit particles sticking to each other, and also limit the damage by shear to the cells or product (Couto and Sanromán, 2006 and Pandey, 1991). Different microorganisms have different tolerances to the frequency and the mixing speed (Mitchell *et al.*, 2006). Usually, a low rotation rate is used (1-15 rpm) although high rates of rotation have also been used. Hardin *et al.* (2002) reported that six different flow regimes could be found in rotating drums: slipping, slumping, rolling, cascading, cataracting and centrifuging. Most commonly, solid state fermentation is performed in rotating drum bioreactors without baffles at relatively slow rotation rates in the slumping flow regime (Mitchell *et al.*, 2006). Losane *et al.* (1985) reported that the rotation speed of the drum is usually 1 rev/min, or when the drum is intermittently rotated a few times per day for a minute at 6 – 16 rev/min or more.

5. Paddle mixers

In a paddle mixer, the solid medium is continuously agitated with a paddle (Durand *et al.*, 2003). The paddle mixer has a number of blades which can make mixing more efficient than the rotating drum; however, the fungal physiology may be destroyed by the high shear rates produced (Bhargav *et al.*, 2008). The paddle mixer can operate with or without force aeration.

Fermenters for submerged culture

The most common types of submerged fermenters used for the industrial applications are stirred-tank fermenters, bubble columns and modification thereof (e.g. airlift reactors) (Garcia-Ochoa *et al.*, 2009).

6). Stirred tank fermenters

A stirred tank bioreactor is a mechanically agitated bioreactor. It is formed by an upright cylinder with baffles to encourage mixing. Typically, only 70 - 80% of the total volume of stirred reactors is filled with liquid (Doran, 2013). Gas-liquid mixing is done by rotating the impeller. Gas dispersion can be increased by changing the configuration of the stirrer and stirrer speed. Rushton turbines or propellers are widely employed in

large scale commercial applications due to high oxygen transfer rates and excellent mixing that can be achieved (Boodhoo *et al.*, 2008 and Garcia-Ochoa and Gomez, 2009). Impeller design is critical for power use and oxygen transfer. The particles in the fermenter may be damaged or destroyed by the impeller or high speed (Doran, 2013). Stirred tank bioreactors have been extensively used for enzyme and pigment production (Schmitt and Blanc, 2001; Nadeem *et al.*, 2009 and Wang *et al.*, 2003), including *Monascus* pigments (Kim *et al.*, 2002 and Dominguez-Espinosa and Webb, 2003). In laboratory studies, the highest yield in a 5 L fermenter with a stirrer with a diameter of 78 mm was obtained at the agitation speed of 500 rpm but the yield decreased at the stirrer speeds over 500 rpm which was directly attributed to shear damage (Kim *et al.*, 2002).

7). Bubble column fermenters and modified bubble columns:

A bubble column is basically a cylindrical vessel with a gas distributor at the bottom. An airlift reactor is essentially a bubble column which has been modified by installing a draft tube to form an internal or external loop to enhance liquid circulation. Both bubble columns and modified bubble columns are widely used in biochemical process industries due to their simple mechanical design, low shear rate, good mixing and low cost. The bubble column fermenters typically are elongated tubes with an aspect ratio of height to diameter greater than 6:1. The air is introduced at the bottom of the tower into either a liquid phase or a liquid-solid suspension and aeration and mixing are achieved by the use of rising bubbles instead of an impeller (Abashar, 2002, Kantarci *et al.*, 2005 and Schmitt and Blanc, 2001). There are low maintenance and operating costs associated with bubble columns due to their compactness and their lack of movable parts (Kantarci *et al.*, 2005 and Schmitt and Blanc, 2001). Bubble columns have been used to produce bakers' yeast, beer and vinegar and in waste water treatment. They have also been used extensively for product formation with filamentous fungi, for example in penicillin production, the production of mycoprotein and citric acid formation (Doran, 2013 and Schmitt and Blanc, 2001).

Mixed solid state and submerged fermentations

Most large scale solid state fermentations use either tray type or drum type fermenters (Pandey, 1991) whereas large scale submerged fermentations typically use

stirred and bubble column fermenters (Garcia-Ochoa *et al.*, 2009). Some processes, however, use a combination of solid state fermentation and submerged fermentation. In the Koji process and in the Moromi process of rice wine and soy sauce production, for example, the fermentation starts with a solid state culture which is then combined with a second stage of submerged culture. Hsu *et al.* (2002) have reported a combination of solid state and submerged cultivations using rice and a monosodium glutamate (MSG) solution. It consisted of a packed absorption of rice at the top and a bubble column with an MSG solution at the bottom. The MSG solution was filtered over the rice grain and recirculated. They proved that the production of red pigment by *Monascus* can be increased by 24% this way compared with standard fixed-bed fermentations.

2.8 Transport processes in bioreactors

Solid state fermentations

To achieve high pigment production rates during the cultivation of *Monascus* cultivation, a good understanding of the transport processes that occur during cultivation is important. A sketch showing the different transport processes and conversions that occur during solid state fermentation is shown in Figure 2.10 for a system with forced convection of air. They can be divided into macroscale transport processes in the bulk of the material between the substrate particles and microscale processes on the surface and within the substrate (Viccini *et al.*, 2001). Fungal growth is mainly on the surface. The processes are complex with simultaneous production of enzymes by the cells and diffusion of enzymes to the bulk of the particles, conversion of the particle material by enzymes, and diffusion of the released nutrients to the cells; transfer of the heat generated by the particles through the chamber wall; exchange of moisture between the air and the particles; and transport of gas (oxygen/CO_2) in the airspace between the particles, across the particles interface and diffusion within the particles.

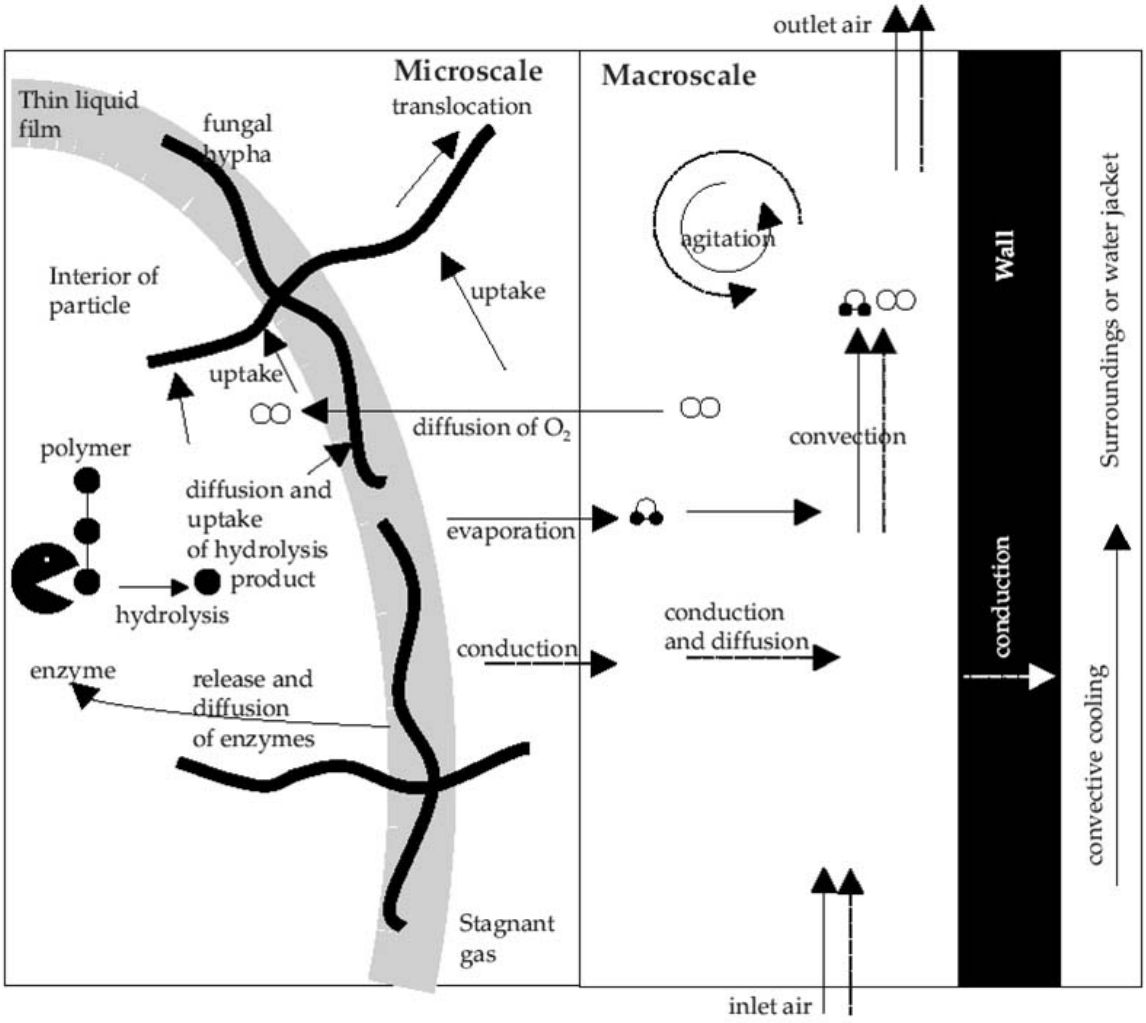

Figure 2.10 Macroscale and microscale transport processes that occur within an SSF bioreactor. (Source: Viccini *et al.*, 2001)

Although, in general, the rate of conversion of substrate is often assumed to be limited by oxygen diffusion (Viccini *et al.*, 2001), this may well be an oversimplification (Mitchell *et al.*, 2003), and it has been argued that local temperature rises due to inadequate heat transfer and the resulting local evaparation of water may be even more important (Mitchell *et al.*, 2003, Rajagopalan and Modak, 1994).

Models such as those proposed by Rahardjo *et al.* (2006), which are based on a rate limitation imposed by the diffusion of oxygen in the fungal biofilm, are able to predict substrate particle degradation in a pseudo-steady state during solid state fermentation, and correctly predicts the decline in oxygen concentration in the bed as the oxygen diffuses through the biomass layer surrounding the particles in the steady state (see Figure 2.11). In the simple reaction-diffusion model it is assumed that oxygen diffusion occurs only from the top of the substrate to the particles. The oxygen concentration in the air and the aerial mycelium layer on the surface is 21%. The oxygen diffuses into the substrate and its concentration decreases as it penetrates the solid substrate due to oxygen consumption and its conversion to product by the fungi. The low oxygen concentration that results because of diffusion limitation is the limiting factor in fungal biomass formation. Where there is no oxygen, the substrate concentration remains constant.

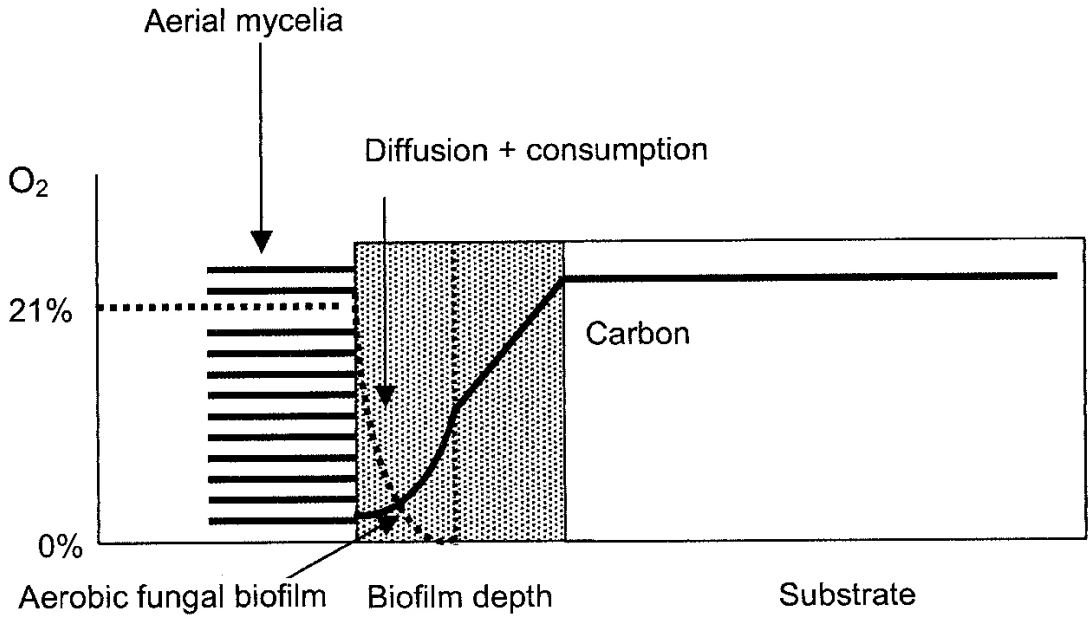

Figure 2.11 Model of oxygen diffusion process in an aerobic fungal biofilm (Source: Rahardjo *et al.*, 2006)

Limitation in oxygen transfer greatly affects production yield in solid state fermentation performance. It is especially important if, during a solid state fermentation, fungal mycelium develops on the solid surface and the voids between the particles are filled with air and water. The oxygen diffusion may then be severely limited. In Figure 2.12, the oxygen concentration profiles in a fungal layer of *Rhizopus oligosporous* growing on the surface of an agar medium containing starch (40 g/l) are shown as an example (Oostra *et al*., 2001). The mycelium grew on the top of the agar and penetrated the solid substrate. After 36 hours, at a depth of about 60 µm oxygen was already depleted.

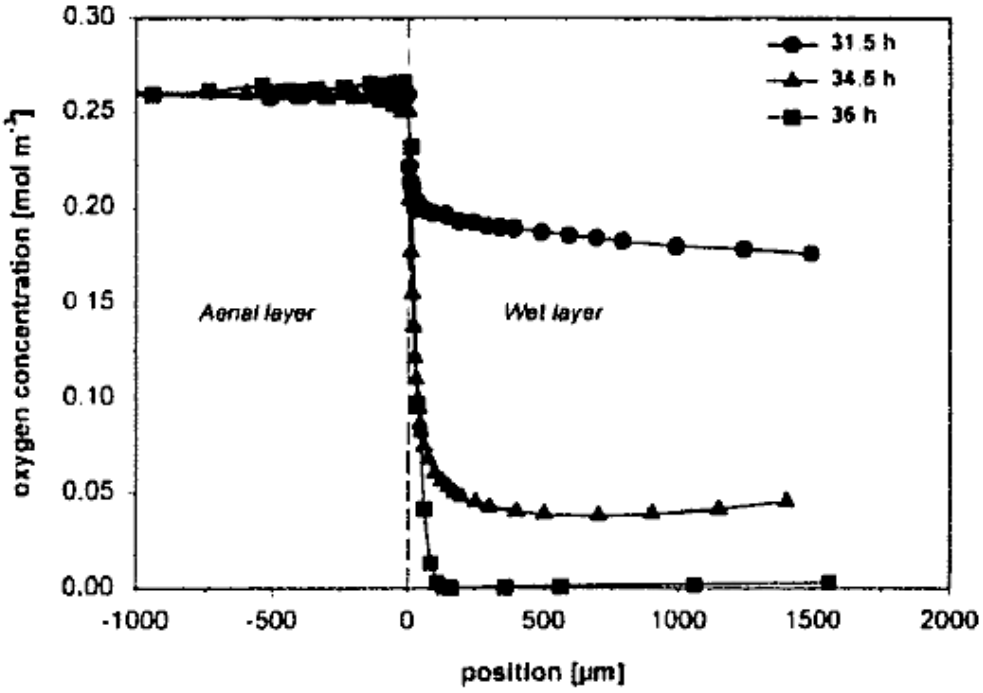

Figure 2.12 Oxygen concentration profiles in a fungal layer of *Rhizopus oligosporous* grown on the surface of an thick agar medium containing starch (40 g/l)
(Source: Oostra *et al*., 2001)

However, limitations in heat transfer are equally important. For example, Rajagopalan and Modak (1994) developed a model which described heat and mass transfer in a tray bioreactor. They showed that oxygen and temperature gradients arise in the substrate layer during the fermentation, leading to localised oxygen depletion and high temperatures within the bioreactor (see Figure 2.13). The raised temperature of the bed can decrease the specific growth rate significantly.

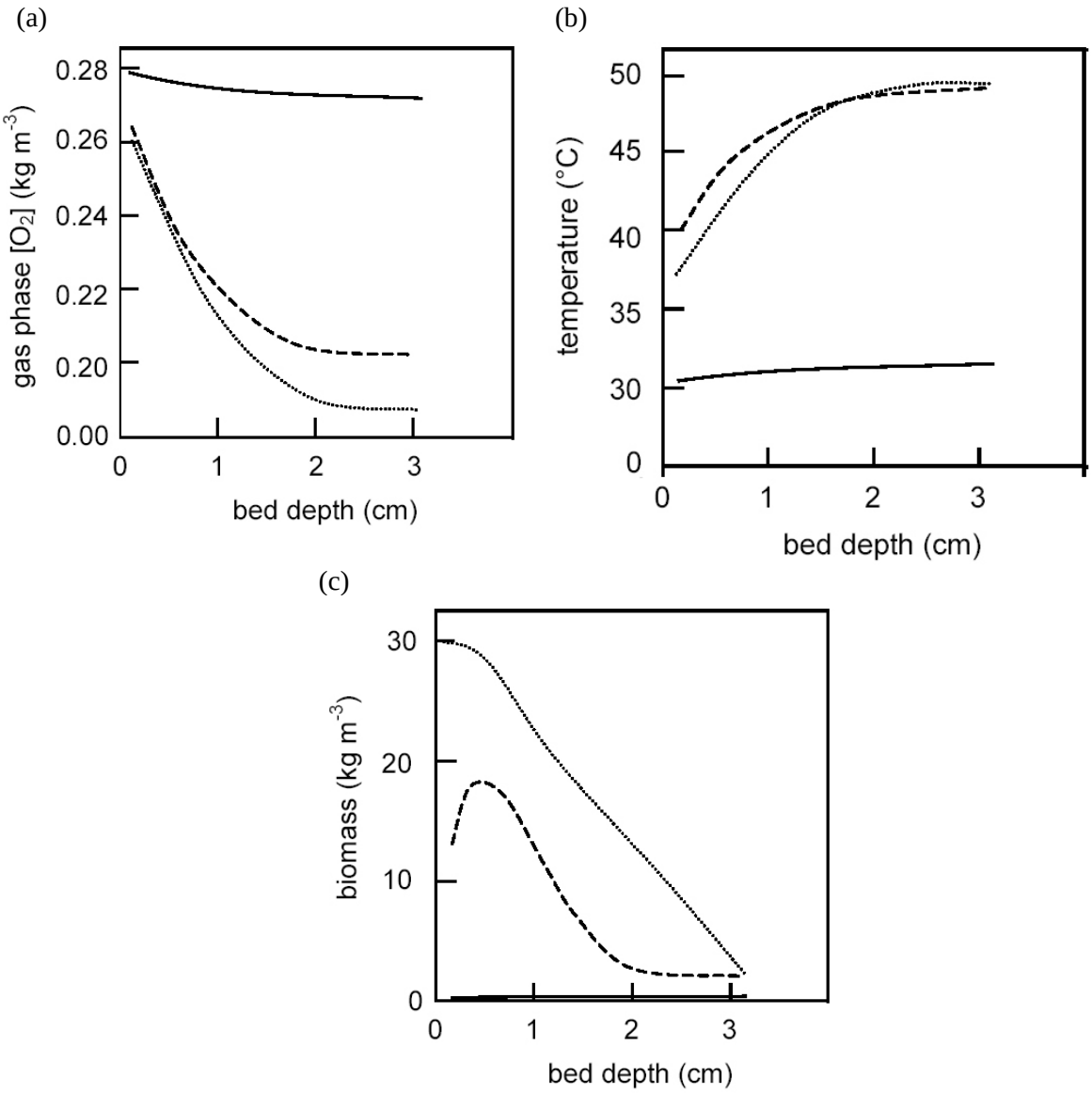

Figure 2.13 Oxygen concentration, temperature and biomass yield model in static bed:

(————) 20 hours, (– – – – –) 60 hours and (·····) 100 hours

Note that the top of the tray in Koji process is represented by zero bed depth and the centre plane corresponds to a bed depth of 3.2 cm.

(a) Oxygen concentration, (b) Temperature distribution and (c) Biomass concentration (Source: Rajagopalan and Modak, 1994)

In their models in a bed of less than 1.6 cm height the highest, biomass levels were reached at the optimum temperature, but when the bed was 3.2 cm high, the incubation temperature had to be adjusted below the optimum temperature to optimize production because the bed was heated up too much by endogenous heat production (Rajagopalan and Modak, 1994). When a bed of over 6.4 cm height was used, it was impossible to maintain any part of the bed near the optimum temperature for growth.

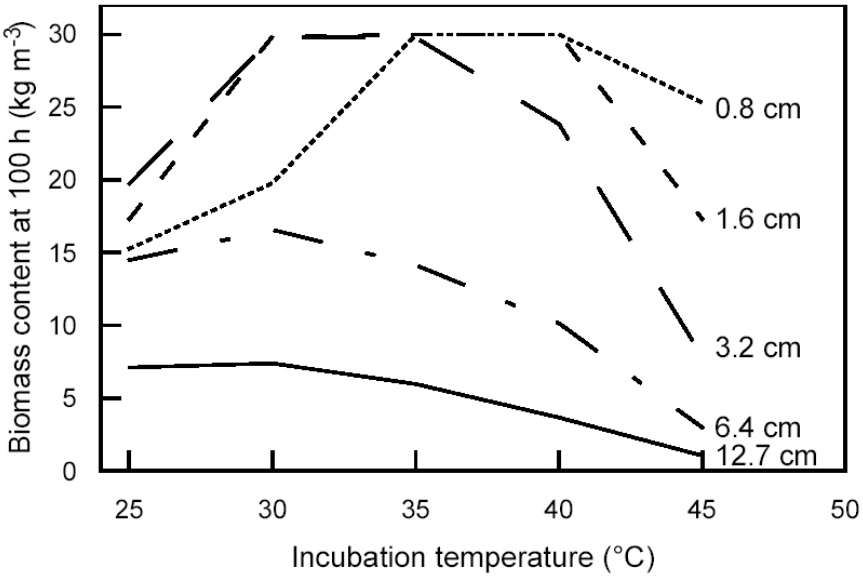

Figure 2.14 Model of the effects of the incubation temperature and bed height on biomass content (Source: Rajagopalan and Modak, 1994)

The heat production in the bed leads to local evaporation in the bed even when humidified air is used. Models incorporating both heat production and evaporation aimed at controlling moisture content during solid state culture have been written (Sangsurasak and Mitchell, 1998, Nagel *et al.*, 2001, Sauceda-Casteneda *et al.*, 1997 and Mitchell *et al.*, 2003).

Submerged fermentations

Transport problems during submerged fermentation are well understood. Control of moisture levels is obviously not a problem, and heat transfer is usually straightforward. The main limitation is related to the oxygen transport. The transport of oxygen from air bubbles to the cells can be divided by a number of steps and resistances, as shown in Figure 2.15. Gas solubility in water is low, and dependent on the temperature, pressure, liquid concentration and occurrence of chemical reactions in the medium. In general, gas solubility in electrolyte solutions is smaller than gas solubility in pure water. Liquid resistances surrounding the bubble control the overall transfer rate and are the rate-limiting step in most aerobic bioprocesses. The overall rate of oxygen transfer can be described by

$$\frac{dC_L}{dt} = k_L\, a \left(C^* - C_L\right) \tag{2.21}$$

k_La = volumetric oxygen transfer coefficient

C^* = saturated oxygen solubility

C_L = the actual dissolved oxygen concentration

The k_{La} consists of two terms: the oxygen transfer coefficient (k_L) and interfacial area between bubble and liquid per unit volume (a). The oxygen uptake rate by the micrororganisms (OUR) is the rate of oxygen consumption from microorganisms. It is a parameter that can be used to evaluate the rate at which metabolic processes take place from microorganisms. Under steady state conditions, the rate of oxygen transfer across the bubble-water interface equals the oxygen uptake rate (OUR) and hence.

$$OUR = q_{O_2} X = \frac{\mu_g X}{Y_{X/O_2}} = k_L a \left(C^* - C_L\right) \tag{2.9}$$

$$\frac{dX}{dt} = Y_{X/O_2} k_L a \left(C^* - C_L\right) \tag{2.10}$$

Where q_{O_2} = specific uptake rate of oxygen

X = cell concentration

Y_{X/O_2} = yield coefficient on oxygen

The oxygen uptake rate (OUR) depends on the organism under study. Typical values are are 40 to 200 mM O_2/h. Often the rate at which oxygen can be supplied in the fermenter is much lower than the maximum rate at which oxygen is consumed by the microorganism. The culture is then oxygen limited. The volumetric oxygen transfer coefficient (k_La) is then a critical parameter for the control of biomass and product yield (Shuler and Kargi, 2002). Many factors must be taken into account when determining the k_La and oxygen need. These include the homogeneity of aeration, geometrical parameters of the bioreactor, composition of fermentation medium, and strain of microorganism (Hölker *et al.*, 2004 and Garcia-Ochoa and Gomez, 2009).

In stirred tanks, aeration is achieved by bubbling air bubbles through the medium which are then dispersed by the stirrer. Higher stirred rates generally lead to more bubble breakup, higher k_La's and hence more oxygen transfer from the gas phase

to the medium. However high stirrer speeds can cause shear rate leading to damage to the mycelia. For example, in an experiment on the culture of *Monascus* performed by Kim *et al*. (2002), agitation of fermentation broth at 500 rpm and 700 rpm gave the same mycelium form but pigment formation was higher at 500 rpm than 700 rpm. Using a helical ribbon instead of a six-bladed turbine for red pigment production by *Monascus spp.* could increase pigment yield up to 2.5 times (Jůzlová *et al.*, 1996, Mohamed *et al.*, 2012 and Wang and Lin, 2007). Similar results were obtained by Dominguez-Espinosa and Webb (2003) during the culture of *Monascus* in a stirred tank reactor at different speeds of agitation. Changes of aeration rate at constant agitation speed hardly affected the pigment productivity whereas an increase in the agitation speed increased the amount of shear rate in the reactor which affected both cell morphology and pigment production (Dominguez-Espinosa and Webb, 2003). Large pellets were formed at 200 rpm whilst at agitation speeds of 333 and 480 rpm the size of the pellets was reduced and the amount of loose mycelia increased (Dominguez-Espinosa and Webb, 2003).

Oxygen transfer in bubble columns is similar to that in stirred fermenters, but bubble columns rely on the pumping action of the bubbles as they rise for the mixing action. Bubble columns generally have been found to be less suitable for pigment production because they have a poor mixing capability. It can however be improved by modifications: *Monascus* pigment production in a modified bubble column with wire-mesh draft tubes was 80% higher than that achieved using a normal bubble column (Jůzlová *et al.*, 1996, Mohamed *et al.*, 2012 and Wang and Lin, 2007).

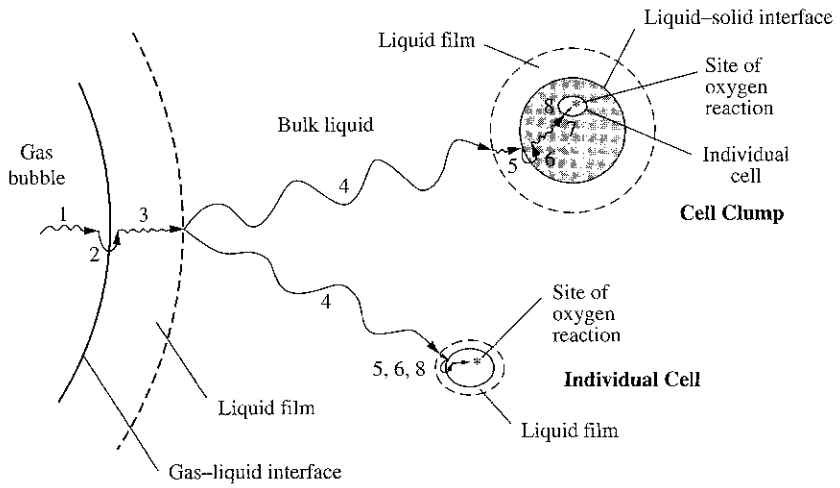

Figure 2.15 Steps and resistance of oxygen transfer from bubble to cell.
(Source: Doran, 2013)

2.9 Scale up of bioreactors

Scale up is a procedure for the design and construction of a large scale system on the basis of the results of experiments with small scale equipment. Normally, it involves transferring data obtained in laboratory and pilot-plant equipment to industrial-scale production. Fermentation processes which can be scaled up without any difficulty are a rarity, because of the enormous increase in the volume or bulk of medium, which is up to 250-1000 times more (Lonsane *et al.*, 1992 and Garcia-Ochoa and Gomez, 2009). The opposite of scale up is scale down; a typical application of scale down is to develop small scale model systems of industrial processes so process conditions can be studied in the lab (Lonsane *et al.*, 1992).

In Western and European countries, solid state fermentation was neglected after the 1940's as efforts were directed towards the development of submerged fermentation (Lonsane *et al.*, 1992). The theoretical foundations of submerged fermentation are therefore already strong, and they are still being extended. The engineering principles of solid state fermentation bioreactors by contrast only began to be developed in the late 1980s (Mitchell *et al.*, 2006). Scaling up of the solid state fermentation system is therefore still difficult, and the development of large scale solid state fermentation processes is hampered by the lack of simple tools for the design of bioreactors (Hardin *et al.*, 2000). Scale up of solid state fermentation is usually done based on geometric similarity and trial and error techniques (Lonsane *et al.*, 1992), but increasingly model-based approaches are also being used ((Sangsurasak and Mitchell, 1998, Nagel *et al.*, 2001, Sauceda-Casteneda *et al.*, 1992 and Mitchell *et al.*, 2003).

Some researchers have developed simple rules for scale up of solid state fermentations. For example, Hardin *et al.* (2000) compared three strategies for scale up of aerated solid state fermentations in a rotating drum based on the fact that evaporative cooling can be of help in controlling the temperature and moisture content of the substrate during a solid state fermentation (Lonsane *et al.*, 1992, Mitchell *et al.*, 2006 and Saucedo-Castaneda *et al.*, 1992). The strategies were: (1) maintain geometric similarity and use a constant volume of air per total volume of bioreactor per minute (vvm); (2) maintain geometric similarity and use a constant superficial velocity (the volumetric air flow rate divided by the overall cross-sectional area of the drum); and (3)

maintain geometric similarity, and vary the air flow rate to keep the ratio of heat generation to heat removal constant. None of the rules were very successful, but scaling up with a constant ratio of heat generation to heat removal gave the best results overall.

In submerged culture, the problems of scale up in a fermenter are associated with the behaviour of gas and liquids in the fermenter and the metabolic reactions of the organisms (Aiba *et al.*, 1973). Bubble columns have been successfully used to grow shear sensitive filamentous cells in the lab but they do not always work satisfactorily at larger scales. For example, Wang and Lin (2007) reported that bubble columns often suffer from blockages at the bottom and low rates of oxygen transfer due to the high viscosity of the medium. Scaling up stirred-tank fermenters can also be problematic because the ratio of the interface area to the volume becomes smaller at a large scale (Wang and Lin, 2007, Garcia-Ochoa and Gomez, 2009). For shear sensitive particles the higher tip speeds of the impeller in the stirred tank reactor at larger scales can cause the particles to break into smaller pieces. To overcome this impeller speeds can be reduced, but this can lead to poor mixing and dead zone formation of substrate and waste products (Fu *et al.*, 2003).

In practice, physical terms which are useful for scaling up submerged fermentations are (see also Table 2.1):

a. Power consumed per unit volume of liquid, *P/V*

$$\frac{P}{V} \propto n^3 D_i^2 \tag{2.5}$$

b. Liquid circulation rate inside the vessel, *F/V*

$$\frac{F}{V} \propto n \tag{2.6}$$

c. Impeller tip velocity, *v*

$$v \propto n D_i \tag{2.7}$$

d. Reynolds number (where ρ = liquid density and μ = liquid viscosity)

$$\frac{n D_i^2 \rho}{\mu} \propto n D_i^2 \tag{2.8}$$

Where P = power consumed for agitation

V = liquid volume

F = pump rate of impeller

n = rotation speed of impeller

D_i = impeller diameter

ρ = liquid density

μ = liquid viscosity

Table 2.1 Relationships between properties for scale up (Shuler and Kargi, 2002)

Property / Scale-up criterion	Small scale 80 *l*	Large scale 10,000 *l*			
		Constant P/V	Constant n	Constant nD_i	Constant, $nD_i^2\rho/\mu$
P	1.0	125	3125	25	0.2
P/V	**1.0**	**1.0**	25	0.2	0.0016
n	1.0	0.34	1.0	0.2	0.04
D_i	1.0	5.0	5.0	5.0	5.0
F	1.0	42.5	125	25	5.0
F/V	**1.0**	0.34	**1.0**	0.2	0.04
nD_i	**1.0**	1.7	5.0	**1.0**	0.2
$nD_i^2\rho/\mu$	**1.0**	8.5	25	5.0	**1.0**

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The criteria that are most often used in industry for the scale up of submerged fermentations are: constant specific power input, *P/V* (30% of use); constant volumetric mass transfer, k_La (30%); constant impeller tip speed of the agitator or shear rate (20%); and constant dissolved oxygen concentration (20%) (Garcia-Ochoa and Gomez, 2009). The scale up ratio is typically 1:10 for processes but lower ratios decrease the risk of unexpected performance during scale up.

Power input per unit volume of broth (*P/V*), volumetric oxygen transfer coefficient (k_La) and dissolved oxygen concentration (C_{O_2}) all strongly affect the final product concentration (Aiba *et al.*, 1973). The k_La gives a measure of the ability of the system for oxygen transfer in the liquid phrase and its value is affected by the oxygen consumption by the microorganisms. The impeller tip speed gives a measure of the shear rate in the fermenter and has an upper limit during the culture of filamentous organisms. Kim *et al*. (2002) reported a maximum tip speed of 2.0 m/s for a *Monascus* culture.

2.10 Scaling down with miniature bioreactors

Scaling down in bioreactors can provide experimental systems that work at a smaller scale that can duplicate the environment that exists at large scales (Kumar *et al.*, 2004). At the smaller scale, parameters can be tested more quickly and less expensively than at the production scale and proposed process changes for existing processes evaluated. Scaling down can reduce labour intensity and material costs, achieve product improvement, allow one to perform many cell cultivations in parallel and achieve highly efficient agitation and aeration due to the high mass transfer capabilities at the smaller scale because of the increased surface to volume ratio (Betts and Baganz, 2006, Kumar *et al.*, 2004 and Garcia-Ochoa and Gomez, 2009). This allows rapidly metabolizing, high-cell density microbial cell cultivations to be supported and increases the amount of the product that such bioprocesses can yield. It can be expected that growth kinetics and product formation, optimised at miniature-scales, can be scaled up quantitatively.

Small scale bioreactors can be divided into small bioreactors, miniature bioreactors, also called minibioreactors (MBRs) and microbioreactors. Small bioreactors are small standard bioreactors; typically they have volumes of 100 ml - 2.5 L. Miniature bioreactors are scaled down versions of bioreactors, and typically they have smaller volumes, ranging from ca. 0.1 ml to approx. 100 ml (Betts and Baganz, 2006 and Kumar *et al.*, 2004). Microbioreactors are very small bioreactors which have a volume of several 100 microlitres or less (Schäpper *et al.*, 2009). An example of a microbioreactor is shown in Figure 2.16. This type of reactor seems very promising for future screening applications. Sampling is very difficult and analysis is therefore mainly done on-line (Kumar *et al.*, 2004).

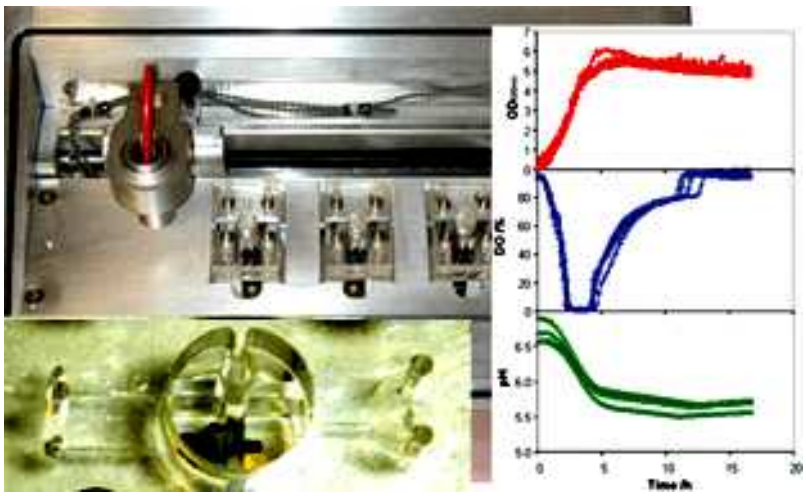


Figure 2.16 A microbioreactor with a 5 µl working volume was integrated with optical sensors for the measurement of OD, dissolved oxygen and pH. The vessel consists of a 5 mm diameter round chamber with two connecting channels for culture inoculation. The body of the microbioreactor was fabricated from polydimethylsiloxane (PDMS) as this is biocompatible and transparent. Dissolved oxygen and pH can be detected using optical fibre sensors. Observed OD, dissolved oxygen and pH were similar to those of a 500 ml bench scale bioreactor. (Source: Szita *et al*, 2005**)**

Many studies of solid state fermentations have been done at a small scale but less at a miniature scale, and to our knowledge none at a microscale. Solid state fermentations at a large scale generally have big problems with heat and mass transfer, and the use of small scale bioreactors significantly reduces these problems. The transfer rates of oxygen, carbon dioxide and water, mixing speed, thickness of substrate bed and temperature in the bioreactor depend on the type and design of the bioreactor used (Bhargav *et al.*, 2008). Typical small scale bioreactors used for solid state fermentation are column bioreactors and drum bioreactors.

Small, miniature and microbioreactors have all been used for submerged fermentations. MBRs used for submerged cultures can be classified into shaken devices (Erlenmeyer flasks, microtiter plates), test tubes, stirred fermenters, bubble columns and other miniature devices such as spin flasks and spin tubes. Shaken devices to culture microorganisms were used as early as the 1940s (Kumar *et al.*, 2004). Shaken flasks are the most commonly used culture vessels in the bioprocess development but have limitations in respect to the control of important process variables such as pH and oxygen. Miniature stirred bioreactors (MSBRs) and miniaturized bubble column reactors (MBCRs) have been developed as alternatives to shaken systems. Stirred

bioreactors provide a high degree of freedom for increasing mixing and mass transfer by increasing stirred speed and gassing rate. Furthermore, stirred systems are perfusion systems in terms of sampling, data collection, on-line monitoring and control (Kumar *et al.*, 2004). Stirred bioreactors comprise various mini- and microbioreactors and specially designed small-scale devices for microorganism cultivation (Kumar *et al.*, 2004). Small-scale bubble columns can be classified between the shaking flask and the stirrer-tank reactors (Betts and Baganz, 2006, Duetz, 2007, Hessel *et al.*, 2008, Garcia-Ochoa and Gomez, 2009, Ge *et al.*, 2006, Lamping *et al.*, 2003, Micheletti and Lye, 2006, Weuster-Botz *et al.* 2001 and Weuster-Botz *et al*, 2002).

(A) Shake flasks

Erlenmeyer flasks, also known as shake flasks, are widely used as simple bioreactors for the screening of microorganisms as well as in basic studies for microbial process development (Altembach-Rehn *et al.*, 1999). They can be agitated using either orbital or linear shaking and can be housed in a temperature-controlled cabinet (Betts and Baganz, 2006). Millions of experiments are conducted annually world wide in shake flasks for screening and culture optimization. The size of shake flasks can range from 10 to 500 ml and be made from borosilicate glass or (hydrophilic) plastic materials. They may be equipped with or without baffles (Betts and Baganz, 2006 and Kumar *et al.*, 2004). Liquid medium typically fills about 20% of the nominal flask volume. Generally, the maximum shaking frequency used is around 350 rpm (Altembach-Rehn *et al.*, 1999). Shake flasks rely on surface aeration for oxygen transfer; oxygen transfer rates are generally quite low. There are many factors which affect oxygen transfer in shake flasks such as vessel size, filled volume, geometry of baffles and shaking frequency (Betts and Baganz, 2006). Betts and Baganz, 2006 reported overall volumetric mass transfer coefficient (k_La) values in shake flasks of up to 0.04 s^{-1}.

(B) Microtitre plates (MPTs)

Microtitre plates (also called microwell plates) were first introduced in 1951 (Betts and Baganz, 2006). Microtitre plates have been used for decades in enzyme-linked immunosorbent assays for medical diagnostics, the pharmaceutical industry, etc. They can be used as miniature shaken bioreactors and are particularly suitable in the

screening stages cell-line evaluation for process development (Betts and Baganz, 2006). They provide a large number of parallel reactors at a miniaturized scale with identical shapes and fluid dynamic characteristics. Several thousand samples may be handled in a short time. Standard well numbers in each plate vary from 6, 12, 24, 48, 96, 384 to 3456 wells (Betts and Baganz, 2006). The bottoms of the wells may be round or flat in shape and the wells deep or shallow. Culture volumes used in microtitre plate vary from 0.025 to 5 ml. (Betts and Baganz, 2006 and Kumar *et al.*, 2004). Betts and Baganz, 2006 reported that oxygen transfer in microtitre plates is in proportion to the shaking amplitude and the frequency and the inverse with filled volume. k_La values in 96 well plates are up to 0.06 h^{-1} .

Figure 2.17 Microtiter plate (Source: Lammer *et al.*, 2009**)**

(C) Test tubes

Test tubes are useful for developing inocula for small scale fermentations and screening strains in primary screening experiments. They are available in various volumes ranging from 2 to 25 ml. They can be made from glass or polymers. The openings of the tubes are fitted with a cotton or plastic foam plug for maintaining the sterility of the culture. Usually, 20% of the total volume is filled with the culture medium (Kumar *et al.*, 2004).

(D) Spinner flasks and spin tubes

Spinner flask bioreactors are designed to grow animal cells (Kumar *et al.*, 2004 and Betts and Baganz, 2006). A typical spinner flask is made of borosilicate glass, has a stirrer containing the magnet and side arms with screw cap closures. The side arms can be used for inoculation, sampling, medium inlet and outlet, pH probes, exchanging the air and etc.

(E) Miniature stirred bioreactors

Miniature stirred bioreactors are similar to conventional stirred bioreactors, but at a miniature scale. Their performance is similar to that of conventional bioreactors. Temperature, pH and dissolved oxygen can be controlled and maintained at desired levels as in conventional bioreactors. The vessels are made of borosilicate glass or plastics such as polycarbonate, polymethyl methacrylate or polyetheretherketone. They can have ports for the insertion of probes for temperature, pH and dissolved oxygen (Betts and Baganz, 2006 and Kumar *et al.*, 2004. Figure 2.18 shows a miniature stirred bioreactor of 18 ml working volume constructed of stainless steel and pyrex and equipped with optical probes to measure dissolved oxygen and pH on-line (Betts and Baganz, 2006). High oxygen transfer capacities can be achieved in MBRs with k_La values of around 0.3 s^{-1} (Garcia-Ochoa and Gomez, 2009 and Kumar *et al.*, 2004). Betts and Baganz (2006) reported k_La values of over 0.2 h^{-1} (12 ml volume) and as high as 0.44 h^{-1} (8 ml volume) for an MBR agitated at 2300 rpm.

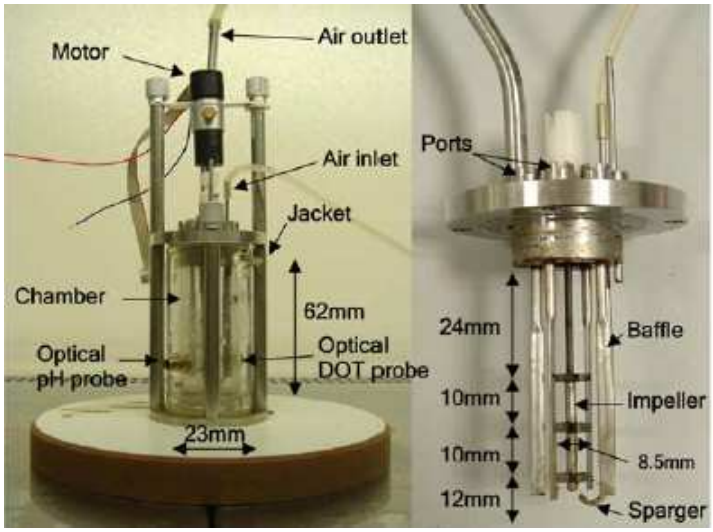


Figure 2.18 Miniature stirred bioreactor (Source: Betts and Baganz, 2006**)**

(F) Miniature bubble column bioreactors

Bubble columns utilise gas sparging instead of agitation as a means of promoting mixing and oxygen transfer for cell cultivation (Betts and Baganz, 2006). In bubble columns, the power supplied by the compressed gas disperses the gas in the liquid and mixes the medium at the same time (Altembach-Rehn *et al.*, 1999). Their construction is simple but mass transfer is limited to k_La values of about 0.3 s^{-1} due to the restriction of power input. A commercial device with multiple reactors including pH measurement and control is available (Kumar *et al.*, 2004). For example, Weuster-Botz *et al.* (2001) reported that volumetric oxygen transfer in a small scale bubble column with working volume of 200 ml is up to 0.15 $h^{-1}$and Doig *et al.* (2005) reported k_La value in 2 ml microtitre plates (MTP) with porous membranes (frits) is up to 0.06 h^{-1}.

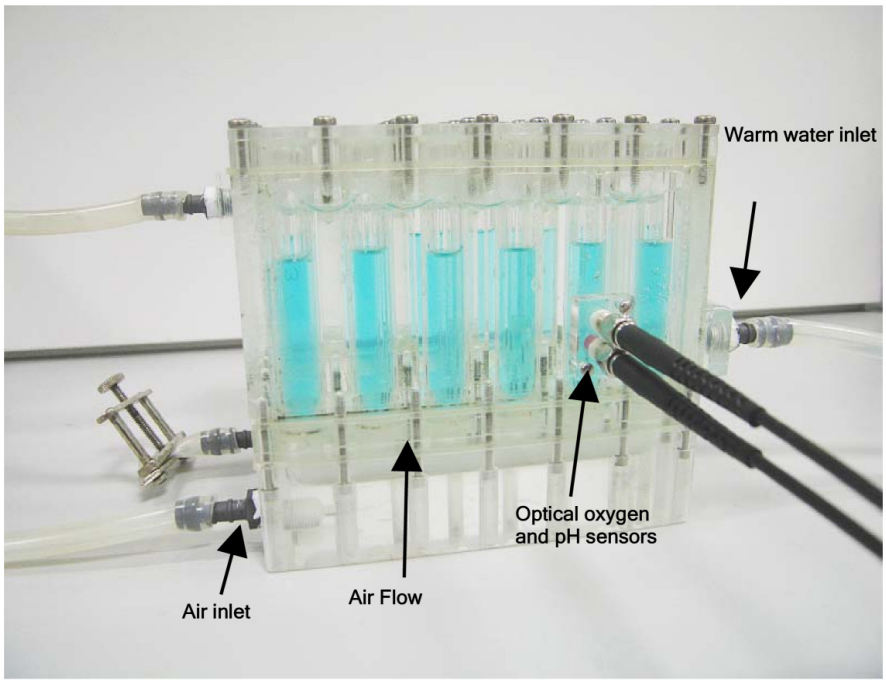


Figure 2.19 Miniature bubble column bioreactors (Source: Betts and Baganz, 2006**)**

Angkak from rice contains citrinin whereas adlay angkak has no citrinin. Solid state fermentation is more effective in enhancing secondary metabolite production. The study will therefore start with *Monascus* pigment production from adlay through solid state fermentation. Figure 2.20 gives an overview of the experiments conducted and reported in this thesis. The work can be divided into product development and process development. During product development, chemical and biological supplements will

be added during solid state culture to optimize the maximum pigment yield. It will be explored whether mixing will make a more homogeneous product and the acoustic vibration technique will also be applied to see whether it enhances pigment formation. The details of experimental settings will be given in chapter 3 and the results and discussion will be given in chapters 4 to 9. Chapter 10 will give overall conclusions and suggestions for future work.

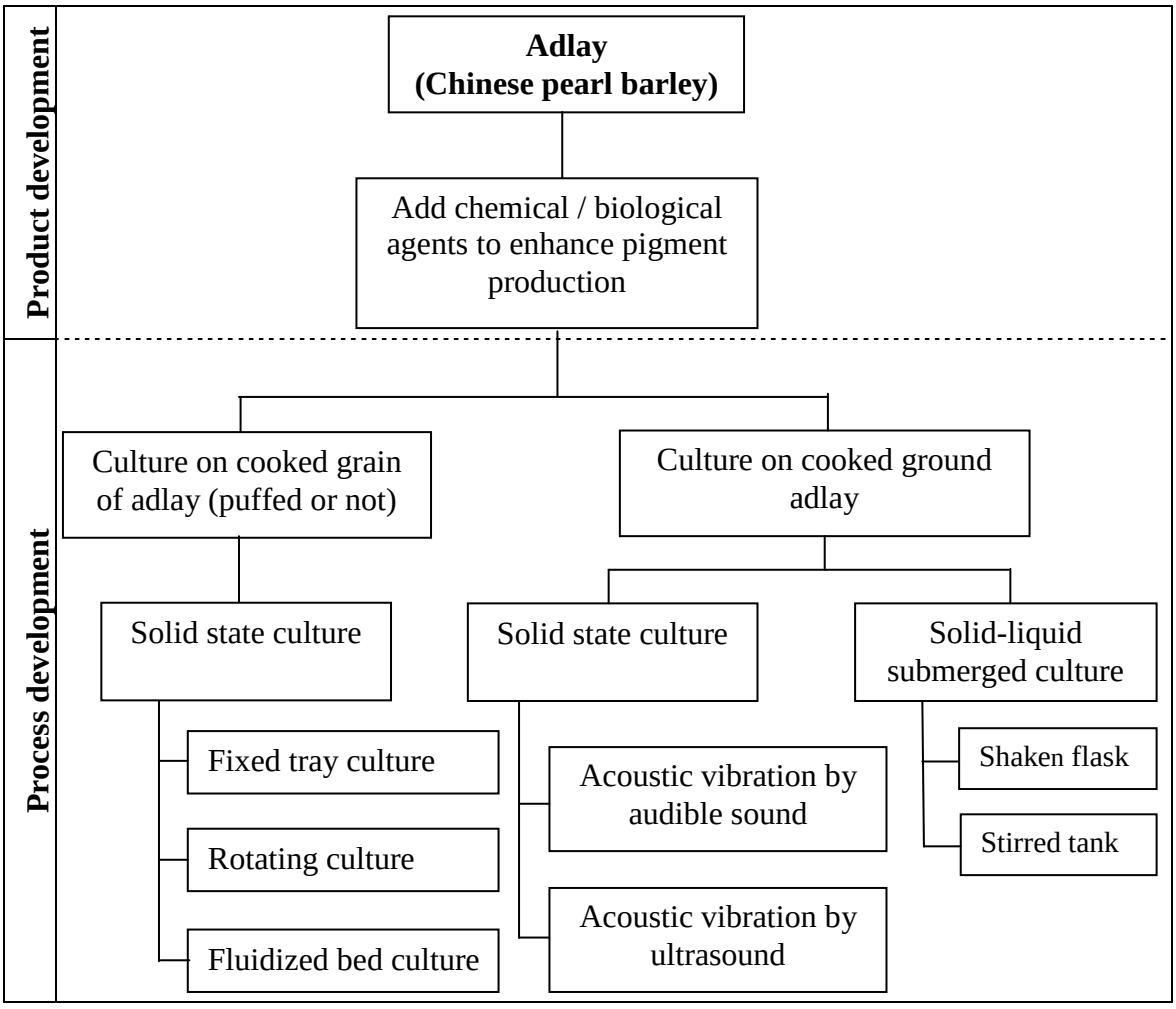


Figure 2.20 Diagram of product and process development of adlay angkak

CHAPTER 3

MATERIALS AND METHODS

3.1. Introduction

This chapter provides information about material, methods and the experimental setup which was used throughout the work in the thesis. More specific detailed information will be given in each chapter. It can be divided into four parts: microorganism, media preparation, culture methods and analysis.

3.2. Microorganism

Monascus ruber (strain TISTR3006, obtained from Chiang Mai University, Thailand) was used to produce *Monascus* pigment. *Monascus ruber* grew quicker and gave greater pigment yields than *Monascus purpureus* on adlay angkak (Pattangul *et al.*, 2008). Strain preservation and spore formation were performed on Potato Dextrose Agar (PDA). Spore suspensions were prepared with 10^5 CFU/ml as seed cultures (Babitha *et al.*, 2007).

3.3 Media

Thai jasmine rice (Vudhichai Produce Co., Ltd., Green Dragon™, Thailand), polished whole grain Chinese pearl barley (adlay, Heng Cheuny Company, Golden Lily™, China) and puffed adlay (Process barley, Mart Billion Ltd., China) were bought from the Hing Sing Chinese supermarket (Leith Walk, Edinburgh, UK).

To obtain adlay powder, whole grains of adlay were ground with a disc grinder (Buhler Miag type DLFU, Klöckner Moeller AT11-1-I, Bauknecht™ made in Braunschweig, Germany) in the Department of Food Science, School of Life Sciences, HWU). Particle size was around 0.3 mm.

The substrate for solid state fermentation was prepared by mixing whole grain or puffed or ground adlay or rice and distilled water in a container. After that, the material was sterilised at 121°C for 15 minutes in an autoclave. Then, either a 0.5 cm diameter *Monascus* agar plug or spore suspension was added. The material was then incubated at 30°C for three weeks (modified from Pattangul *et al.*, 2008).

The basis for the liquid medium for submerged culture was a nutrient solution containing KH_2PO_4 0.25%, $NaNO_3$ 0.3% and $MgSO_4.7H_2O$ 0.4% in distilled water (w/v) (modified from Chang *et al.*, 2002, Lian *et al.*, 2007, Silveira *et al.*, 2007 and Wang *et al.*, 2009). Adlay powder or glucose was added as the carbon source. A suspension of adlay was sterilised at 121°C for 15 minutes but a glucose solution was sterilised at 110°C for 10 minutes. To initiate fermentation, 2% by volume of spore solution was added to the liquid medium as the inocula. The culture was then incubated at 25°C for two weeks.

3.4 Culture systems

Two different culture methods were used: solid state culture and submerged culture. Erlenmeyer flasks, volumetric cylinders, universal bottles, thin bottom glass bottles and polycarbonate plastic boxes were used for the study of solid state cultures under static conditions. Polycarbonate cylinders were used as chambers for the study of solid state cultures in mixed beds.

3.4.1 *Solid state cultures of Monascus*

3.4.1.1 *Erlenmeyer flask*s

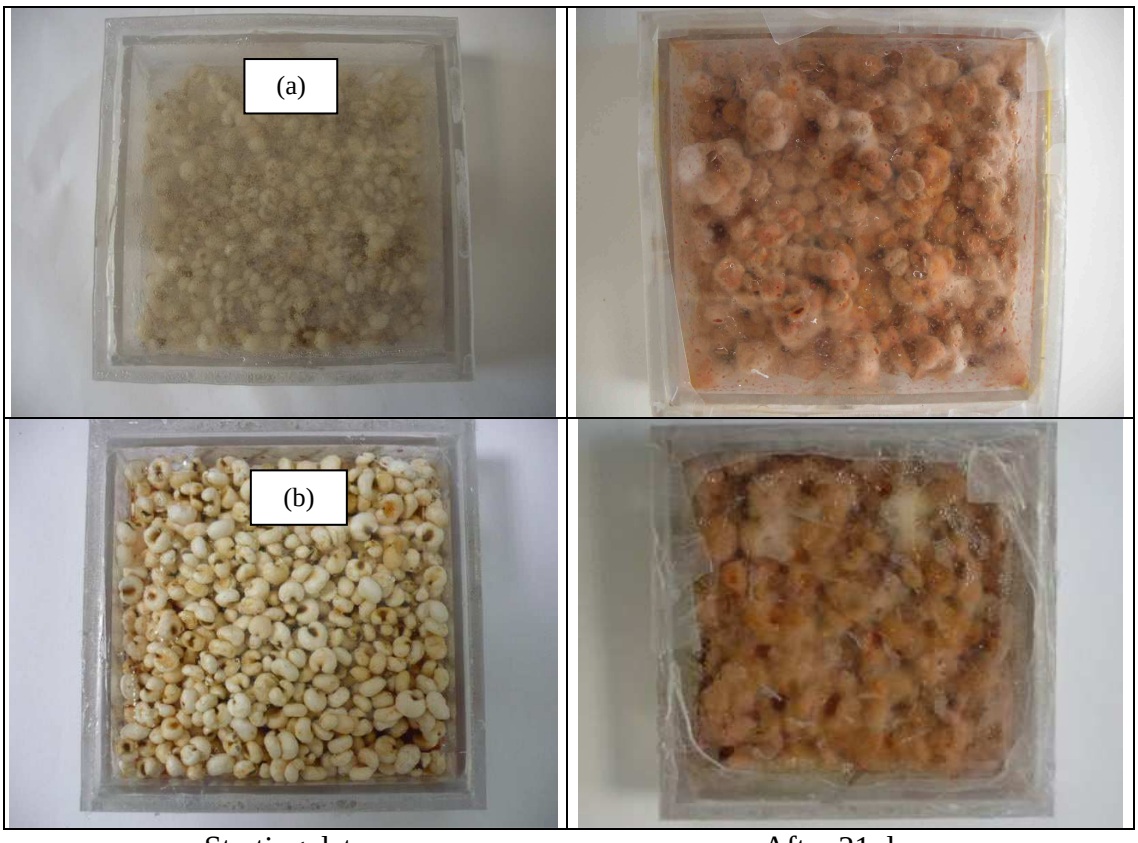
25 g of adlay or rice was put into a 250 mL Erlenmeyer flask and the appropriate amount of distilled water added (typically 25 mL). In some studies nitrogen sources or sugars were also added at this stage. The flask opening was covered with aluminium foil and then sterilised at 121°C for 15 minutes (110°C for 10 minutes if additional nutrients were present). The 250 ml Erlenmeyer flasks were then incubated for three weeks at 30°C.

3.4.1.2 *Cylinders*

The 100 ml volumetric cylinders were used for the study of the depth of penetration of the fungus into a bed of substrate. 50 g of polished whole grain adlay, rice or ground adlay were added to each of the cylinders and 50 mL of distilled water added. The cylinders were covered with aluminium foil and then sterilised at 121°C for 15 minutes. Inoculation was done with a 0.5 cm diameter plug of agar with *Monascus*. The cylinders were incubated at 30°C and the depth of pigment penetration was measured every day for six weeks.

3.4.1.3 *Trays*

Polycarbonate plastic boxes (10cm (W) x10 cm (L) x 2 cm (H)) were made in-house (see in Figure 3.1) and used as tray bioreactors. 15 g of whole grain or puffed adlay were added to the trays and the appropriate amount of distilled water added. In some experiments, sugar and a nitrogen source were also added. The adlay was then sterilised at 110°C for 10 min in an autoclave. 1 ml spore solution was added to start off the fermentation and the tray was kept in an incubator at 30°C for three weeks.



Starting date After 21 days

Figure 3.1 Fixed-tray bioreactor with adlay before and after the culture of *Monascus*
(a) Whole grain adlay (b) Puffed adlay

3.4.1.4 *Universal bottles*

1 g of whole grain or puffed adlay was added to universal bottles, forming a single layer of the grains at the bottom of the universal bottle. The universal bottles were sterilised at 121°C for 15 min in an autoclave. Spores were added and mixed with the adlay with an inoculation loop. After inoculation the bottles were incubated at 30°C.

3.4.1.5 *Rotating drum bioreactors*

Miniaturised bioreactors were constructed as shown in Figure 3.2. Each reactor was composed of 2 chambers. Each chamber was made from 8 cm high sections of 3.5 cm ID polycarbonate tube and 1 cm thick polycarbonate sheet. The first chamber was used for culture and the second chamber as an aeration zone. The aeration zone had a sponge to filter the air on the top. The reactors were connected to low speed 38 mm ironless 12/24V DC motors with spur gearboxes (RS components) which allowed the chambers to be rotated horizontally along their axes. Each motor was powered by a DC power source; the connection to each power source was controlled by a timer (Tempatron Ltd, UK). The timer controlled the time intervals and periods that the chamber was rotated. The bioreactor was continuously rotated at speeds of 0.25, 8 and 16 rpm in an incubator at 30°C for three weeks. In another set of experiments, the bioreactor was used as an intermittent rotating culture. The reactor was rotated at 0.25, 8 and 16 rpm for three minutes, and 1, 3 and 8 times per day. A static chamber was used as a control sample.

Figure 3.2 Rotating bioreactors used for growing *Monascus* on puffed adlay

3.4.2 Submerged cultures of Monascus

3.4.2.1 Shaken Erlenmeyer flasks

100 mL of a nutrient solution with 4% glucose was added to a 250 ml Erlenmeyer flask and sterilised at 110°C for 10 minutes. The flask was then inoculated and incubated on 150 rpm orbital shaker. The temperature in the incubator was controlled at 25°C (see Figure 3.3a).

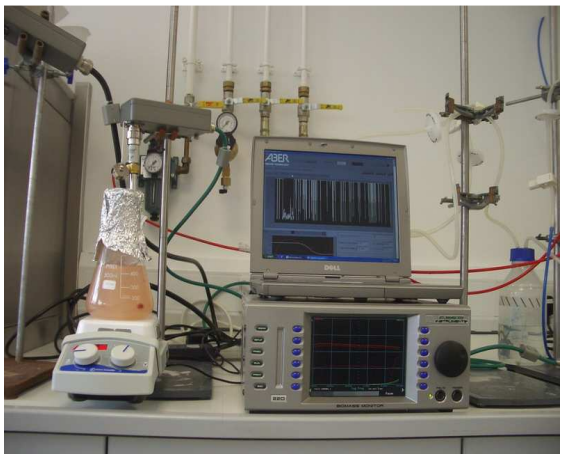
3.4.2.2 Stirred Erlenmeyer flasks

The 500 ml Erlenmeyer flasks were used for the culture with a magnetic stirrer. The 400 ml glucose nutrient solution was put in the flask. It was sterilised and inoculated. The mixing speed was adjusted to approximately 500 rpm, and the flask was incubated for three weeks at 25°C (see Figure 3.3b).

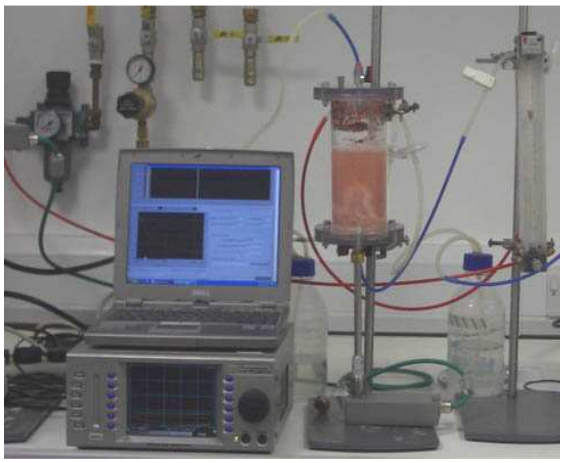
3.4.2.3 Bubble columns

A 1.5 litre polycarbonate plastic cylinder was used as a bubble column (see Figure 3.3c). The bubble column had an ID of 8.5 cm and a height of 30 cm. 1 litre of glucose medium was put in the bubble column reactor. It was sterilised and inoculated with *Monascus* spore suspension solution. Humidified air was bubbled through the reactor. The air flow rate was set at 4 litres per minute. The temperature of the reactor was controlled at 30°C by recirculating warm water around the vessel.

(a) Shaken flask



(b) Stirred flask



(c) Bubble column

Figure 3.3 Some of the bioreactors used for the submerged culture of *Monascus*

3.4.2.4 Miniaturised bioreactors

Miniature bioreactors as shown in Figure 3.4 were made from 8 cm high sections of polycarbonate tube with an inner diameter of 3.5 cm and 1 cm thick polycarbonate sheet. The stirred reactor had four stainless steel baffles, and was stirred with three 2 cm wide, 6 mm high paddle-shaped stirrers. A biomass sensor probe was inserted through the bottom to measure the capacitance on-line. Mixing was either done by shaking or stirring. Shaking was done by placing the reactor on an orbital shaker at 150 rpm. The volume of medium in the reactor was 20 mL. Stirred reactors were stirred using an IKA Labortechnik stirrer. Stirring rates used were 400, 800 or 1200 rpm. The liquid volume in the reactor was 10, 20 or 30 mL.

Figure 3.4 Experimental setups for submerged culture of *Monascus* in stirred (left) and shaken (right) miniaturized bioreactors

3.5 Co-culture of *Monascus* and yeast

Co-cultures of *Monascus* and yeast were done in 250 ml flasks. 25 g whole grain adlay and 25 mL of a nutrient solution (0-10% fructose, glucose or sucrose by weight) were added to the flask. The flasks were sterilised at 110°C for 10 min. 1 mL of suspension of baker's yeast with 10^6 cells/ mL was added, and the inoculated adlay incubated for a week at 30°C. 1 mL of a spore suspension of *Monascus* was then added, and the flasks incubated for three weeks at the same temperature.

3.6 Exposure of Monascus culture to audible and ultrasound

3.6.1 Exposure to audible sound

A thin layer of ground adlay was put in small glass thin-walled bottles. After sterilisation and inoculation the bottles were kept in a 30°C incubator for three weeks. Audible sound with frequencies between 40 and 160 Hz was applied to the bottles for 15 min every day (modified from Aggio *et al.*, 2011, Swamy *et al.*, 2005 and Yang *et al.*, 2005). To generate the sound, a 500 W speaker was used powered by a Thurlby

Thandar Instruments model TG 120 20 MHz function generator and WA 301 wideband amplifier (see Figure 3.5).

3.6.2 Exposure to ultrasound

In another set of ultrasonic experiments, the bottles were prepared the same way but exposed to ultrasound of 1.080 MHz with different powers between 3 and 12 W. the ultrasound was generated by driving PZT piezoelectric transducers (Ferroperm Piezo-Ceramic A/S) at their resonance frequency using a AG series amplifier (T&C Power Conversion Inc, New York) (see figure 3.6). The radius of transducer was 38 mm and its thickness 2 mm. The transducer was put on an aluminium frame which had air space at the bottom. Three bottles were placed simultaneously on the top of the transducer. All bottles were exposed to ultrasound for 5, 10 and 15 min every day for three weeks (modified from Swamy *et al.*, 2005 and Yang *et al.*, 2005).

Figure 3.5 Experimental setup for the vibration of a *Monascus* culture on ground adlay with audible sound

Figure 3.6 Experimental setup for the exposure of a culture of *Monascus* on ground adlay to ultrasound

3.7. Analysis methods

Seven aspects of the process are analysed in the thesis i.e. pigment concentration, moisture content, glucosamine concentration, mechanical properties (hardness, cohesion strength and adhesiveness), capacitance signal, bulk density and bed packing density, and the structure analysis of the grains).

3.7.1 Pigment concentration

Fermented grain (angkak) and fermented broth were dried in the oven at 55°C overnight until the weight was constant. 0.5 g pigment powder (angkak powder) was dissolved with 5 ml 70% (v/v) ethyl alcohol and shaken for eight hours on a reciprocal shaker at 150 rpm. Supernatants were obtained by centrifugation. Optical densities of yellow, orange and red pigments were estimated using a spectrophotometer (Biochem Ltd., WPA spectrophotometer, England) at 400, 470 and 500 nm respectively (modified from Kim *et al.*, 2002, Pattangul *et al.*, 2008 and Yongsmith, 2000).

3.7.2 Moisture content

The moisture content of cooked grain and fermented grain (angkak) was measured by drying a sample in an oven at 105°C until the weight was constant. The moisture content of fermented grain (angkak) was calculated at the constant weight. The results (n=3) are expressed as a percentage (AACC, 1995 and Maritotti *et al.*, 2006).

$$\%\ Moisture = \frac{Moisture\ loss\ in\ grain}{Orginal\ weight\ of\ sample}$$

$$= \frac{Orginal\ weight\ of\ sample - Dried\ weight\ of\ sample}{Orginal\ weight\ of\ sample}$$

3.7.3 Mechanical properties

The mechanical properties (hardness, cohesion strength and adhesiveness) of cooked adlay (whole grain and puffed adlay) and its fermented product were assessed using a texture analyser (Zwick/Roell type Boo-FB0.5TS, Zwick GmbH & Co, made in August-Nagel-Straβe, Germany). Hardness (N) means the maximum compression force (positive force) on the material when a plunger moves in close tolerance within the sample cylinder (Ramesh and Srinivasa Rao, 1996). Stickiness was shown in cohesion strength and adhesiveness. The cohesion strength (N) means the maximum drag force (negative force) on the material. The adhesiveness (mJ) means the accumulated work from negative force and travel distance.

3.7.4 Biomass concentration: capacitance

To determine the biomass concentration on-line and offline, an Aber Instrument model 220 Biomass Monitor was used (see Figure 3.7). The capacitance signal at the probe was measured by taking capacitance data at 25 different frequencies in the range 100 kHz - 20 MHz. The data were then averaged (minimally 30 datapoints per given frequency).

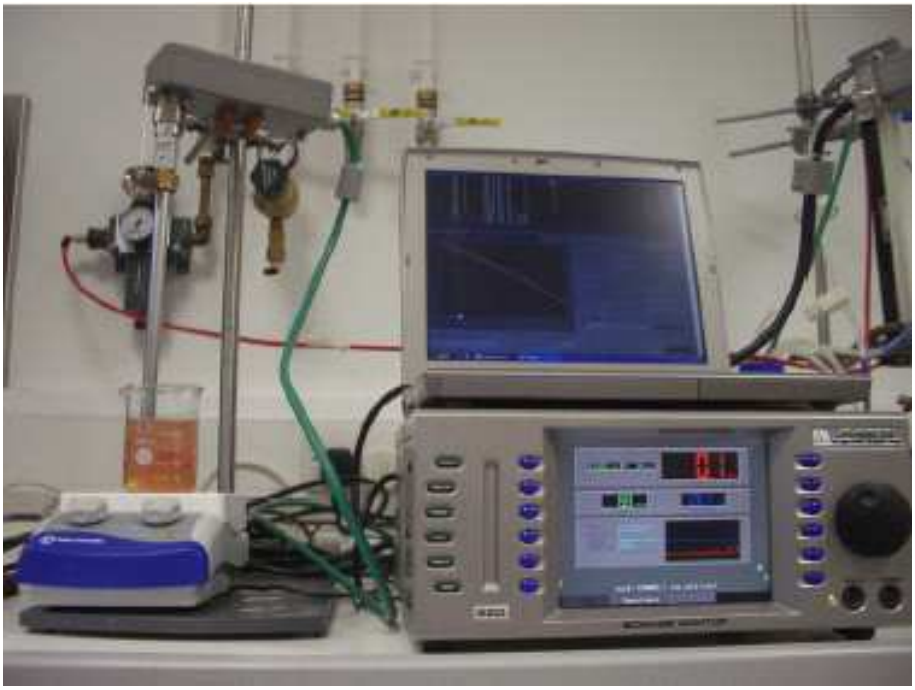


Figure 3.7 The Aber Instruments model 220 Biomass Monitor

3.7.5 Biomass concentration: Glucosamine

Fungal biomass was also estimated by determining the N-acetyl glucosamine concentration in the sample. N-acetyl glucosamine is found in the cell wall of fungi (Carvaiho *et al.*, 2006 and Babitha *et al.*, 2007). The glucosamine concentration is therefore considered to be representative of the fungal cell concentration in foods. First, a 0.1 g from a pigment powder sample was subjected to acid hydrolysis by dissolving it in 5 mL 5 M HCl for 16-18 hours until the solution was clear. The acid hydrolysis sample was then mixed with 1 mL acetylacetone reagent and incubated in a bath of boiling water for 20 minutes. After cooling, 1.5 mL ethanol was added followed by the addition of 1 mL of Ehrlich reagent. After that, the optical density was measured at 530 nm against a reagent blank. N-acetyl glucosamine obtained from Sigma-Aldrich was used as the standard (Babitha *et al.*, 2007).

The relation between biomass cells and capacitance in submerged culture was determined by measuring the glucosamine concentration and capacitance intentsy in different cell concentrations. Then, the off-line of adlay concentration from 0 to 12% weight by volume affected by the capacitance signal was measured by the biomass monitor.

3.7.6 Bed packing density and Bulk density

Bed packing density was determined experimentally by filling a container of known volume (V, liters) and mass, packing the substrate in a manner identical to preparation for the fermentation, and reweighing the filled container. The difference between the masses of the container when it is packed with substrate and when it is empty is the packed mass of the bed (m_p, g) (modified from Mitchell *et al.*, 2006).

$$Bed\ packing\ density,\ \rho_b = \frac{Total\ bed\ mass}{Total\ bed\ volume} = \frac{m_p}{V_{total}}$$

Bulk density is probably one of the most common and widely used of the bulk characteristics (McGlinchey, 2005). The bulk density of a material is simply the mass of material divided by the volume that it occupies (McGlinchey, 2005, Rhodes, 2006 and Shamlou, 1988). The bulk density of grain and flour was determined by measuring the weight of a known volume of sample. Samples were poured into a graduated cylinder, gently tapped ten times and filled to 250 ml. Results are expressed as g/l (modified from Maritotti *et al.*, 2006).

3.7.7 Microscopic analysis of the grain structure

The shape and size of whole grain adlay and puffed adlay grains before and after cooking and after fermentation were measured using a Leica microscope with a JVC camera. This camera was interfaced with an Aquinto 4ai docu image program in a personal computer. Images were saved in JPEG file format. Results (n = 15) were expressed in mm.

To analyse the progression of pigment production in the puffed and whole grain grain adlay, grains were were cut in two and images taken using a stereomicroscope (Steady-T, Ceti, Belgium).

To analyse smaller structures within grains different types of grain were cut into three parts (two outer shell parts and one cross section). Each part was put on carbon loaded tape and aluminium stubs. Before mounting, all samples were assigned a reference number, written underneath the stub. Pictures were then taken using a Scanning Electron Microscope (SEM, FEI model Quanta 3 D FEG) at an accelerating voltage of 20 kV. FEI-xT microscope server software controlled the SEM and provided facilities for image grabbing and analysis.

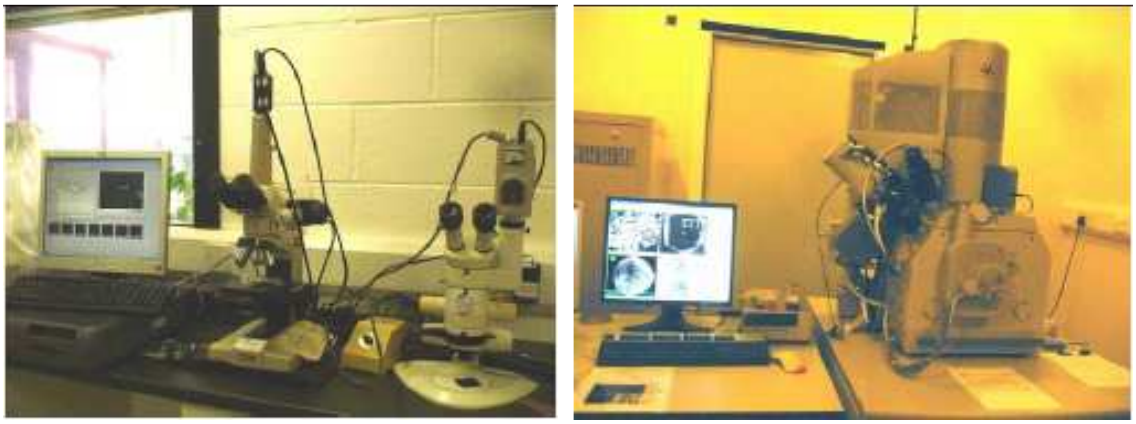


Figure 3.8 Microscope and Scanning Electron Microscope used for analysing grains

CHAPTER 4

COMPARISON OF THE CULTURE OF *MONASCUS* ON A STATIC BED OF RICE AND ADLAY

4.1. Introduction

Colourants are essential tools in the foodstuff industry manufacturing process. Food pigments are used extensively as food additives to enhance sensory response. Adding colour to food improves the presentation of the food, and therefore promotes sales. Food colourants may be natural or synthetic. Most of the natural dyes are extracts from plants or are produced by microorganisms. Pigment molecules include carotenoids, melanins, flavins, quinines and more specifically monascins, violacein, phycocyanin and indigo (Dufossé *et al.*, 2005). The number of permitted synthetic colourants has been decreased because of undesirable toxic effects including mutagenicity and potential carcinogenicity. Scientific research has focused on the development of new food pigments from natural sources (Sabater-Vilar *et al.*, 1999 and Watanabe and Terabe, 2000).

One of the oldest pigments is red koji or angkak, obtained by the fermentation of rice with the fungus *Monascus*. The red pigment produced by the fungus *Monascus* has been widely used in Asia for centuries as a food colourant in the processing of alcoholic beverages, red soybean curd, and fermented products (meat, fish and vegetables). New food applications have also been found, like the colouring of sausages, hams, surimi and tomato ketchup (Dufossé *et al.*, 2005, Silveira et al., 2007, Suh *et al.*, 2000). *Monascus* species can produce at least six major pigment products including the yellow pigments monascin ($C_{21}H_{26}O_5$) and ankaflavin ($C_{23}H_{30}O_5$); the orange pigments rubropunctatin ($C_{21}H_{22}O_5$) and monascorubrin ($C_{23}H_{26}O_5$) and the red pigments rubropunctamine ($C_{21}H_{23}NO_4$) and monascorubramine ($C_{23}H_{27}NO_4$) (Jung *et al.*, 2003).

Rice, scientifically named *Oryza sativa L.*, is a cereal grass from the family Gramineae. It has been cultivated extensively in warm climate regions for more than 11,000 years and is a staple food consumed throughout the world, particularly in Asian

countries. The Chinese have used fermentation with a range of microorganisms to convert agricultural commodities into food for centuries. Red yeast rice, also known as red Koji or Hongqu, consists mainly of non-glutinous rice, red yeast, and by-products of the fermentation. *Monascus* on steamed rice has been used for more than 600 years for producing wines and fermented food products (Ma *et al.*, 2000). Angkak is a long-established food ingredient for Asian consumers, however it is still forbidden in Europe and the USA due to the fact that the mycotoxin citrinin may occur in some batches (Dufossé *et al.*, 2005).

Currently there is a trend in angkak research to change from rice as a raw material to other agricultural products such as Chinese pearl barley, corn, cassava, groundnuts, jackfruit seeds, palm kernels, oats, sorghum, soybean, tamarind seeds, and wheat (Babitha *et al.*, 2007, Dufossé *et al.*, 2005 and Teng and Feldheim, 2001) in order to increase pigment yield and reduce mycotoxin concentration in the pigment. Adlay (Chinese pearl barley) is one of the most interesting raw materials as pigment can be produced with a very low citrinin content (less than 1 µg per gram pigment) (Yang *et al*., 2004). Adlay, scientifically named *Coix lachrymal-jobi L.*, is a grass crop from the Gramineae family. Other general names for this are adlay in the Philippines, Job's tear in Australia, mayuen in China, and sila in Fiji. It is widely planted in many countries including Brazil, China, India, Japan, Philippines, Taiwan and Thailand (Hu *et al*., 2007, Shih *et al.*, 2004 and Yang *et al*., 2004). It is used in traditional Chinese medicine as a nourishing food, due to its high nutritional value. In addition, to this it has alleged medicinal properties and has been used in the treatment of warts, chapped skin, rheumatism and neuralgia; it has also been alleged to have antitumour activity (Yang *et al.*, 2004).

The culture of *Monascus* on adlay could be done by solid state fermentation or submerged fermentation. The rate of production in solid state cultures depends on the rates of oxygen consumption and diffusion. Oxygen diffusion in air is much easier than in water due to oxygen having a diffusion coefficient in air (at temperatures typically found in culture, 25 – 35°C) that is 8,000 – 10,000 times higher than in water (Bird *et al.*, 2002). The diffusion of oxygen in solids is even slower due to the molecules in solids being more stationary, and oxygen diffusion in grains, which contain significant amounts of solids and water, can be expected to be much smaller than in air. The oxygen demand from fungi depends on type of nutrient sources, water content, pH and temperature. Pigment production decreases if oxygen consumption is limited by the rate

of oxygen diffusion. As a result of the limitation of oxygen diffusion in the bed the layer of substrate used in tray fermentations cannot be very deep; typically a thin layer of substrate from 2 to 15 cm is used. (Aido *et al.*, 1982, Ito *et al.*, 2011, Malathi and Chakraborty, 1991 and Mitchell *et al.*, 2000). It has been suggested that the layer of substrate in a tray layer should be no more than about 1.5 – 2.0 cm thick. A culture in a layer of less than 2 cm suffers less from the limitation of oxygen transfer and the oxygen concentration at the bottom is nearly at zero (Couto and Sanromán, 2005 and Muniswaran *et al.*, 2002). Moreover, Babitha *et al.* (2006) studied the effect of *Monascus* pigment production in different particle sizes (0.1 – 0.6 mm) of jackfruit in the thin layer substrate bed (5 g of seed powder was taken into 250 mL Erlenmeyer flasks) and the particles between 0.3 and 0.4 mm gave the maximum pigment yields.

Adlay is a novel type of substrate, and in the literature there is no information about oxygen limitation in a bed of adlay and the depth at which pigment can be produced. In this chapter, pigment production in a static bed of rice and adlay is reported. Both whole grain adlay and ground adlay were used.

4.2. Results and discussion

4.2.1. Effect of particle size on *Monascus* solid state culture

In the first set of experiments, a comparison was made of the growth of *Monascus* in the solid state culture in stationary flasks on whole grain and ground grain (see 3.4.1.1 in Chapter 3). The effect of different particle size was studied on rice (Jasmine rice) and adlay (Chinese pearl barley). The flasks were incubated at 30°C in an incubator over a period of three weeks. The results are shown in Figure 4.1.

Although *Monascus* could grow on both whole grain and ground grain, there were clearly large differences in the nature of the growth of the fungus on both materials. Even though whole rice and adlay grain had significant differences in shape (cylindrical and spherical, respectively) and size, the effect of this was much smaller than the effect of grinding. Growth and pigment production by *Monascus* on whole grain was significantly better than on ground grain. Pigmented *Monascus* could be seen to cover all of the grains in the flask after about two weeks of incubation, whilst *Monascus* could still not penetrate to the bottom of the flask after three weeks if grown on ground rice or adlay.

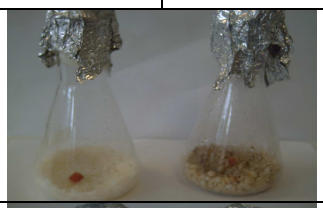
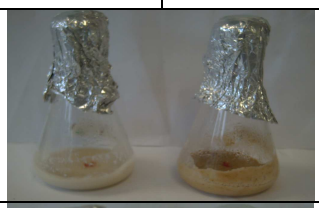
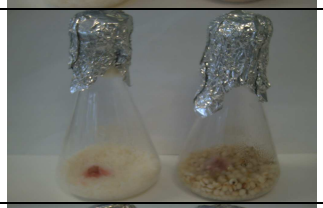
No. of day	*Monascus ruber* TISTR3006 growth images			
	Whole Grain		Ground Grain	
	Rice	Adlay	Rice	Adlay
Starting date				
3 days				
6 days				
9 days				
12 days				
15 days				
18 days				
21 days				

Figure 4.1 The appearance of *Monascus* growth on whole grain and ground grains of rice and adlay

Grinding gave the particles a higher surface area and it supported higher fungal growth and pigment production rates. The result showed that *Monascus* grew on whole grain better than on ground grain. The reason for the lack of penetration of *Monascus* is most likely the change in the nature of the ground material after it is cooked. Gelatinisation of the starch during the cooking process can make the particles sticky and cause them to aggregate and conglomerate. If the particles are small then they can form a layer of material without any voids between the particles for air to penetrate. This caused a limitation of the exposure of the bed material to air when the bed material was ground. This will lead to diffusion limitation of oxygen into the bed. In contrast, if the particles are large, then voids between particles are likely to be formed that are large enough for air to move into the bed. Oxygen limitation is therefore less likely.

4.2.2. Depth of pigment production in a bed of rice or adlay

To investigate oxygen transfer limitation during a solid state culture in more detail, and to determine the depth to which *Monascus* might penetrate into the bed, a number of experiments were done in which different materials were placed in a 100 ml cylinder, and the depth of penetration of the fungus (as determined by the depth at which pigment could be seen to be produced) determined (see 3.4.1.2 in Chapter 3).

In the first experiment, whole grains of adlay were placed in a 100 ml cylinder (see Figure 4.2), cooked, inoculated with *Monascus* and incubated at 30°C. The penetration depth of *Monascus* pigment into the bed of whole grain adlay was measured over a period of six weeks, and then the cylinder was removed and the concentration of the pigment and the moisture level in the cylinder were measured. The results can be seen in Figure 4.2. The penetration depth of pigment can be seen to increase for four weeks, and then stop increasing. Pigment content within the cylinder depended on the depth. At the top of the cylinder (0 – 4 cm), the highest concentrations of coloured pigments were found. This top layer gave yellow, orange and red pigment concentrations of 98.7 ± 8.7, 28.0 ± 3.3 and 28.3 ± 3.7 units per gram respectively. At 4 cm depth the yellow pigment concentration had declined to 71.2 ± 8.0 units per gram, whilst the orange and red pigment concentrations remained relatively constant at 27.0 ± 2.2 and 25.2 ± 4.5 units per gram respectively.

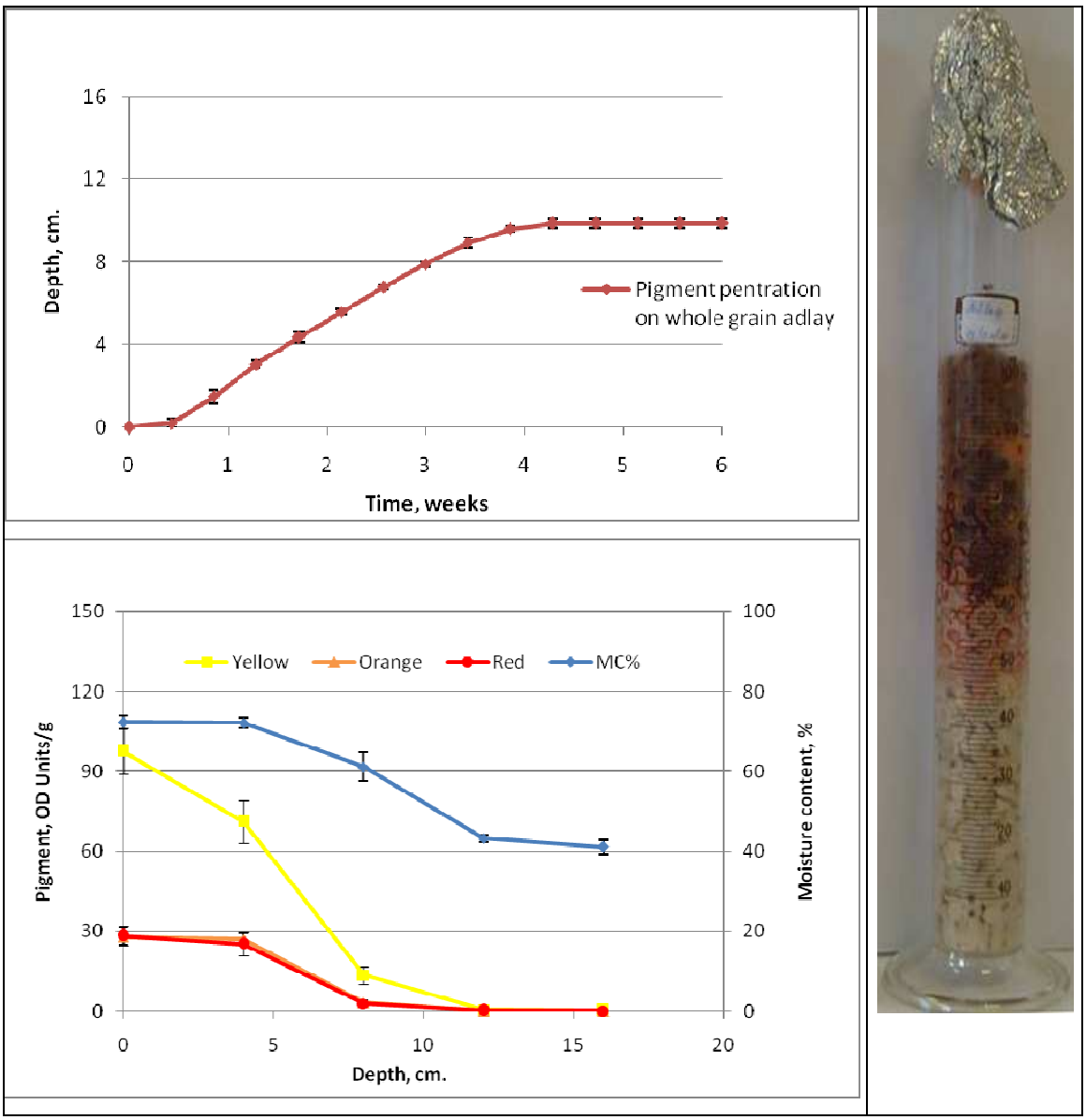


Figure 4.2 Distribution of pigments and moisture after six weeks of culture of *Monascus* in a column of whole grain adlay.

Between 4 and 8 cm the pigment concentration declined rapidly. Between 8 and 12 cm little pigment was found. At a depth of more than 12 cm no pigment was produced. Therefore, it can be concluded that a static culture on whole grains of adlay should be no more than 12 cm thick. Beyond this depth there appears to be a complete absence of pigment production. The highest concentrations of pigment are found in the top layer of 4 cm, and the optimum thickness of a layer of whole grain adlay for the solid state of *Monascus* therefore appears to be 4 cm.

The reason for the observed behaviour is most likely to be the limited rate of oxygen diffusion in the bed. On the top surface of a bed, the grain particles are in an

environment rich in oxygen. The fungi can easily take enough oxygen from the air. However, deeper in the bed oxygen is consumed by fungus but not replenished quickly enough by the relatively slow diffusion process. Oxygen concentrations will then decline and pigment production decrease. At some stage it will run out; anaerobic conditions may be created, and pigment production stop altogether.

Figure 4.3 shows the same experiment on oxygen transfer limitation on whole grains of rice. Although the change in the depth of pigment penetration over time and the distribution of pigment over the bed at the depth are similar, there are small differences. The pigment penetration depth continued to increase for more than 4 weeks; it only stopped after 39 days. The depth to which the pigment was produced in the rice was longer than in the whole grain adlay by a distance of 3 cm. The reason for the higher penetration depth on the whole grain rice compared to the whole grain adlay was thought to be related to the fact that rice grains are cylindrical in shape and adlay grains more spherical. This gave the rice a looser characteristic packing. Measurement of the density of a bed of whole grain adlay and rice confirmed this. The density of a bed of whole adlay was 0.98 g/cm^3, that of whole grain rice slightly lower at 0.96 g/cm^3.

The depth of the pigment transport and penetration period in rice was longer than in adlay. The reason for this is not clear but it is arguably because the shape and the composition of the material were different. For example, rice has a cylindrical shape whereas adlay has a spherical shape. Rice therefore has a relatively large surface area for water absorption, and may swell more than adlay during the cooking process, giving a bed with a more open structure.

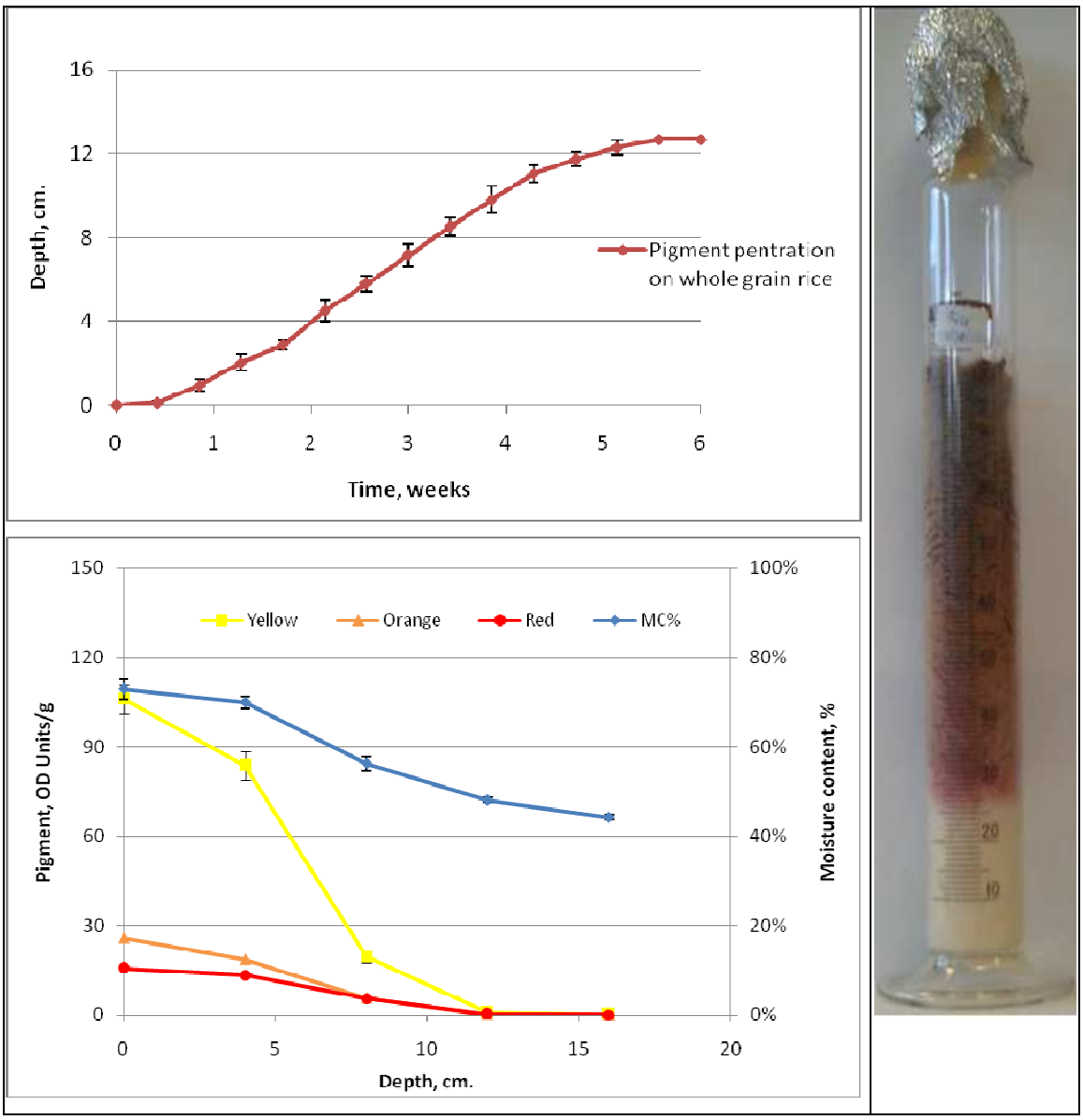


Figure 4.3 Distribution of pigments and moisture after six weeks of culture of *Monascus* in a column of whole grain rice.

In the next set of experiments, *Monascus* was grown on ground adlay. Figure 4.4 shows a diagram illustrating the depth of pigment penetration of *Monascus* during culture on ground adlay. In the first few days, no pigment was produced. Instead, white mycelium was found on the top of adlay. After this short period, pigment started to be produced. After three weeks, the pigment had penetrated to a depth of only 0.9 cm. From three to five weeks the depth had increased only slightly from 0.9 to 1.3 cm. The depth of the pigment penetration then remained stable.

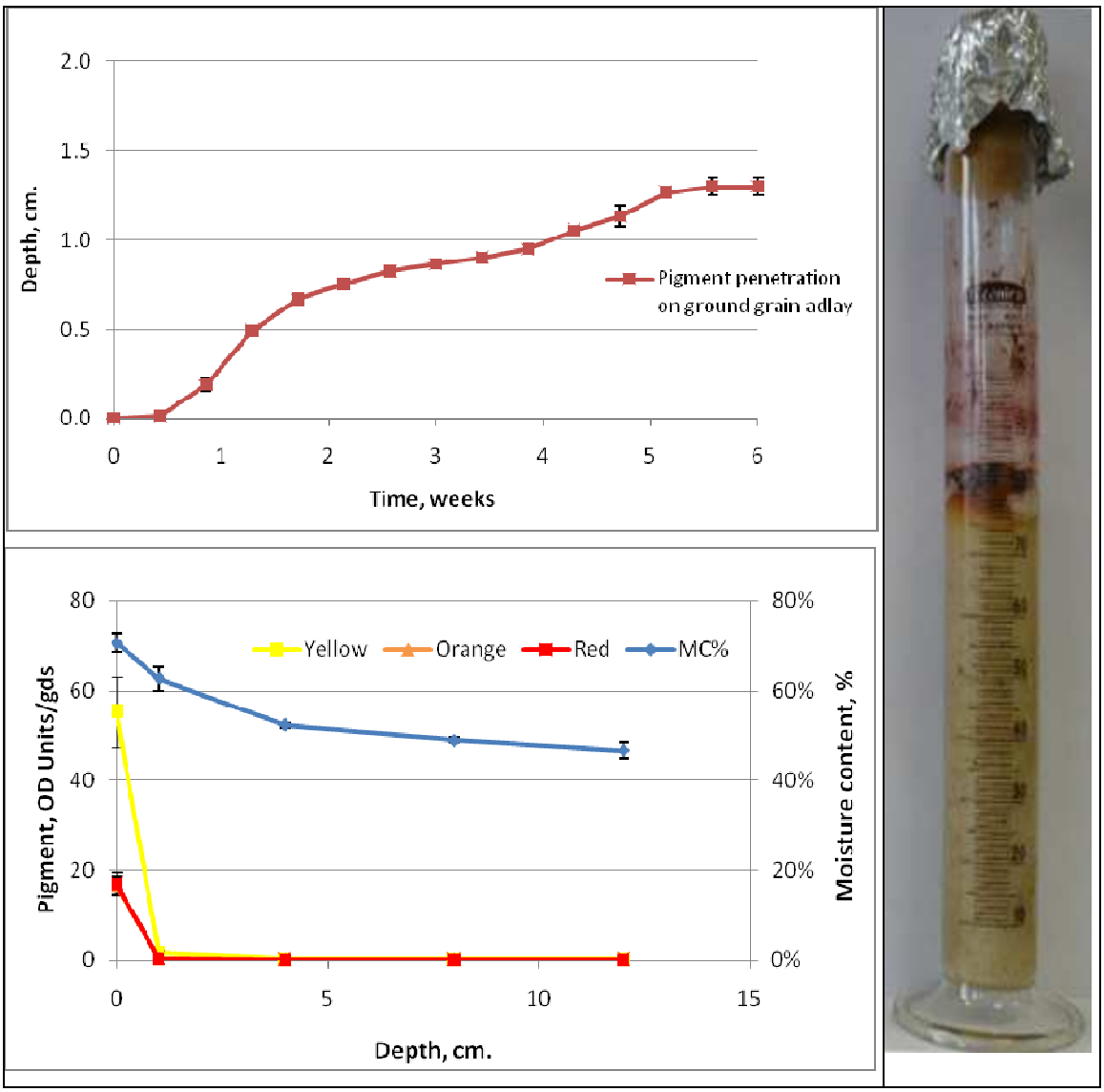


Figure 4.4 Distribution of pigments and moisture after six weeks of culture of *Monascus* in a column of ground adlay(~0.3 mm. with 50% water added)

The top of the bed had the highest pigment concentration (55.2 ± 7.9, 17.5 ± 2.1 and 17.0 ± 2.5 units per gram respectively of yellow, orange and red pigments), but it rapidly decreased to 1.64 ± 0.05, 0.39 ± 0.06 and 0.38 ± 0.05 units per gram respectively of yellow, orange and red pigments at a depth of 1 cm. This indicates that when *Monascus* is grown on ground adlay, oxygen is already highly limiting in a layer of approximately 1 cm thickness. Pigment was only found in the first 1.3 cm, and no pigment was found from 1.3 to 12 cm. Pigment distribution within a cross section was also uneven. Pigment penetrated to a depth of 1.3 cm near the glass surface but near the centre of the column pigment was only produced up to a depth of 0.6 cm. This clearly indicates transport rates were higher at the glass surface. The moisture content in ground

adlay was also quite uneven along the length of the column as it decreased from 70% at the top to 47% at a depth of 12 cm.

When comparing pigment production on ground adlay (Figure 4.4) with that on the whole grain adlay (Figure 4.2), it can be seen that the period during which pigment was produced was shorter when *Monascus* was grown on whole grain adlay (21 days) than when it was grown on ground grain (36 days). The depth to which pigment penetrated was much also much greater on the whole grain (9.5 cm) than on ground grain (1.3 cm).

In theory smaller particles should provide a larger surface area for the fungi to attack and oxygen to diffuse into, and also the limitation of oxygen diffusion inside the particle should have been less because the oxygen has less distance to travel from the surface. The reason for the reduction in pigment production in ground grain is most likely to be the lack of inter-particle spaces (voids) between the particles of ground grain. The grains of adlay tend to stick to each other and agglomerate. The spaces between the particles of ground grain are also very small, and may act like capillaries for any water. The fungal biomass may also block oxygen diffusion through the bed. The end result is a compacted wet solid bed of ground grain which forms a barrier for oxygen transport into the bed. As a consequence, fungal growth on ground grain is very poor, and pigment production remains limited near the surface. Cooked whole grain adlay on the other hand has larger inter-particle spaces, providing the room for oxygen to diffuse more deeply into the bed. Oxygen will still be consumed by the fungi, so oxygen levels will decline deeper in the bed. Oxygen also has to diffuse into the particles, which is a much slower process than in air. The expected pigment production is therefore as in see Figure 4.5.

The density of a bed of whole grain adlay was around 0.98 g/cm^3 and ground grain adlay around 1.25 g/cm^3, confirming that a lack of voids with air in the bed of ground adlay was the cause of the difference in the pattern of pigment production in the two beds.

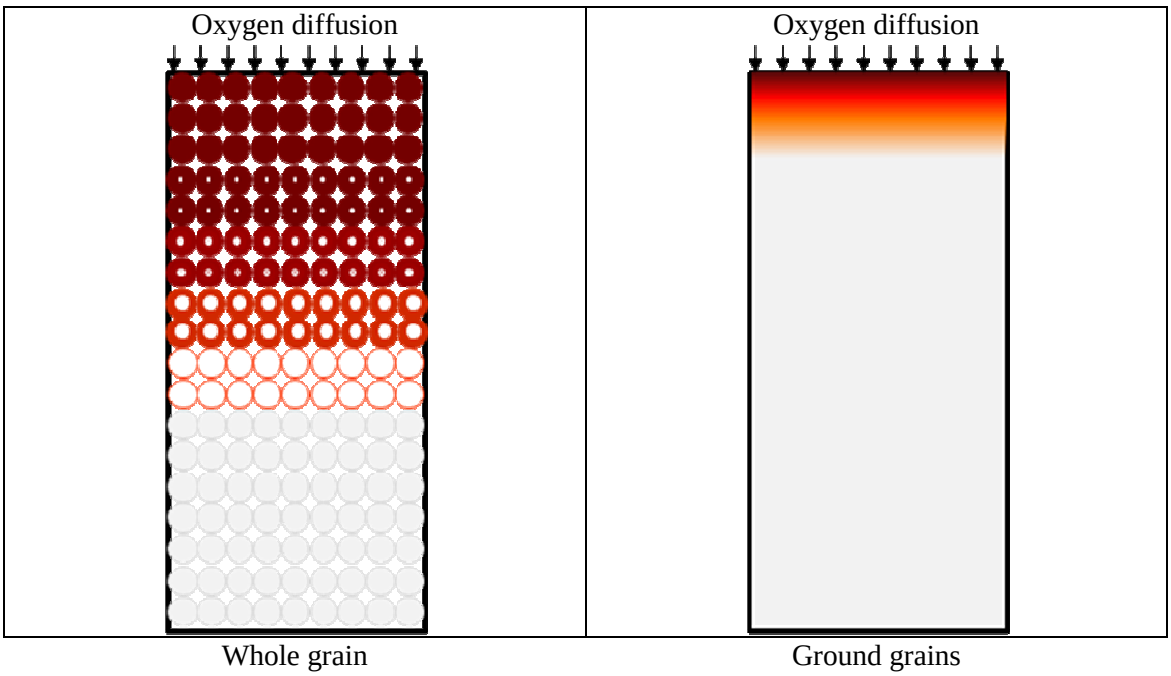


Figure 4.5 Effect of oxygen diffusion on pigment production in static beds of different types of cooked grain

4.4 Conclusions

It was shown that when *Monascus* was grown in the solid state fermentation for adlay pigment production, the growth remain limited to a layer of food material near the top of the bed. The limitation depended on the composition material of the particles and also the particle size. In the bed containing large particles, oxygen can easily diffuse into the voids between the particles but the reaction rate inside the particles is limited by the lower inress of oxygen into the particles by diffusion limitation. A more rapid reaction rate could be achieved in principle by using smaller particles which have a higher surface area to volume ration. However, it was found that when finer particles were used oxygen penetration into the bed difficult was more diffcult due to it having less air spaces in the bed, giving a lower rate of reaction. The lack of air space between the particles was caused by the presence of gelatinised starch, filling the voids between the particles.

The difference between whole grain rice and whole grain adlay was small. When *Monascus* was cultured on whole grain adlay and on whole grain rice at steady state, a maximum depth of pigment penetration was obtained of around 12 cm. High pigment concentrations were found in the first 4 cm at the top, and then decreased. In contrast, the maximum range of pigment penetration in a bed of ground adlay was just 1 cm.

CHAPTER 5

ENHANCEMENT OF *MONASCUS* PIGMENT PRODUCTION USING CHEMICAL AND BIOLOGICAL ADDITIVES

5.1. Introduction

Monascus pigment, angkak, is a natural red colourant which is usually made by culturing *Monascus spp.* on cooked rice. *Monascus spp.* can produce not only red coloured pigments but also yellow and orange coloured pigments (Rosenblitt *et al.*, 2000). *Monascus* pigments are soluble in ethanol and slightly soluble in water. In China, Indonesia, Japan, Korea, the Philippines, Taiwan, Thailand and the United States, it has been used as a natural pigment in marine products, meat products, Chinese red soybean cheese, red rice wine. (Delgado-Vargos and Paredes-Lopez, 2003, Dufossé *et al*., 2005, Erdooğrul and Azirak, 2004, Panagou *et al*., 2003 and Wang and Lin, 2007).

The addition of chemical and biological substances can increase pigment production and yield. Carbon sources which have been added to boost production have included dextrose, ethanol, fructose, galactose, glucose, lactose, lactose, mannitol, maltose, sucrose, xylose, sorbitol, starch and flour. Nitrogen sources which have been added have included ammonium chloride, ammonium sulphate, ammonium nitrate, sodium nitrate, chitin powder, corn steep solid, malt extract, monosodium glutamate (MSG), amino acid, peptone, tryptone, soybean meal and yeast extract (Babitha *et al.*, 2006, Daroit *et al.*, 2007, Lee *et al.*, 2001, Jůzlová *et al.*, 1996 and Silveira *et al.*, 2007). Babitha *et al.* (2006) reported that rice, lactose, cassava starch and sucrose supported pigment production well. Sucrose gave the highest ratio of pigment yield per unit biomass. The addition of MSG, soybean meal, peptone and chitin had a positive impact on pigment production in jackfruit. Jůzlová *et al.* (1996) reported that starch, maltose sucrose and galactose were suitable carbon sources for submerged culture. Lee *et al.* (2001) reported that in submerged culture, glucose and soluble starch were better carbon sources in terms of both growth and pigment production. MSG gave a pigment with a

strongly red colour. Silveira *et al.* (2007) reported that the addition of MSG increased pigment yield in grape waste when a high concentration of peptone (20 g/l) was added. Dufossé *et al.*, (2005) reported that histidine reduced citrinin production and increased red pigment production. Histidine was more successful in reducing citrinin production that any other amino acids.

The use of elicitors can also enhance pigment production. Co-culture of *Monascus* with other microorganisms has been reported before (Shin *et al.*, 1998, Suh *et al.*, 2000 and Wang and Lin, 2007), but only in submerged culture and on agar, and not in solid state culture. Co-culture of *Monascus* with *Saccharomyces cerevisiae* or *Aspergillus oryzae* on agar with a sucrose medium has been shown to increase biomass twices and increase pigment yield 30-40 times compared to monoculture (Shin *et al.*, 1998). Submerged culture with sucrose gave more than a 10-fold increase in pigment (Shin *et al.*, 1998).

From the previous information, it is clear that there is little information in the literature about the enhancement of pigment production by the addition of chemical and biological substances to solid state cultures. Most publications focus on submerged culture, and none at all on the fermentation of *Monascus* on adlay. This chapter therefore focuses on the effects of the use of supplements during the production of highly pigmented adlay angkak with solid state fermentation.

5.2. Results and discussion

This report provides details on experiments on *Monascus* pigment production by *Monascus ruber*. First, *Monascus* growth on adlay was studied to determine the relationship between product yield and time. Following on from these experiments, the focus shifted onto the study of the effect of carbon source, nitrogen source and water content on pigment production during an adlay solid state fermentation. After this, the co-culture of *Monascus* with yeast was investigated. All experiments were prepared in 250 mL Erlenmeyer flask (see 3.4.1.1 in Chapter 3).

5.2.1 *Monascus* pigment production during solid state culture on adlay

As can be seen in Figure 5.1, pigment production on adlay increased over time to reach a maximum after four weeks. Pigment production was low in the first week, and then increased. The change in the rate of pigment production was highest between two and three weeks. After three weeks, the production rate decreased, and the concentration then became constant. The moisture content during the fermentation changed to around 4%. The highest concentrations of yellow, orange and red pigment obtained were 52.5 ± 5.2, 14.5 ± 1.6 and 14.5 ± 1.5 units per gram dry matter, respectively. The data obtained on the culture of *Monascus* on adlay are similar to those described in the literature. For example, Teng and Feldheim (2001) reported that protein content (used as a measure of biomass concentration) and pigment concentration remained low during the first 5 days during the cultivation of *Monascus* on rice, but the residual starch content decreased considerably. Pigment content increased up to 25 days but protein content only increased for 20 days, indicating that pigment production is not linked directly to biomass production.

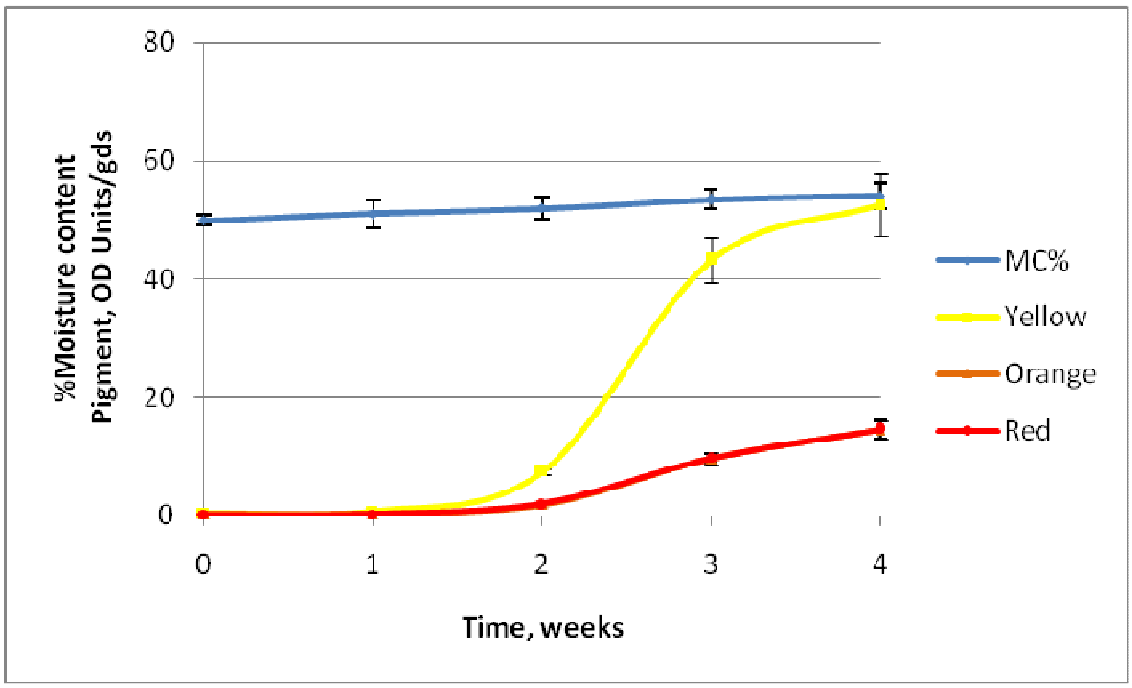


Figure 5.1 Changes in pigment concentration and moisture content during a solid state culture of *Monascus* on adlay

A comparison of a culture on whole grain rice and whole grain adlay, shown in Figure 5.2, shows that after three weeks the pigment concentration in rice was lower than in adlay. The ratio of yellow and red pigments in fermented rice was around 6:1, whereas the ratio of these pigments in adlay was just 4:1. Therefore, an extract solution of fermented rice was orange in colour, whilst an extract solution of fermented adlay was red. The moisture contents in both materials were similar.

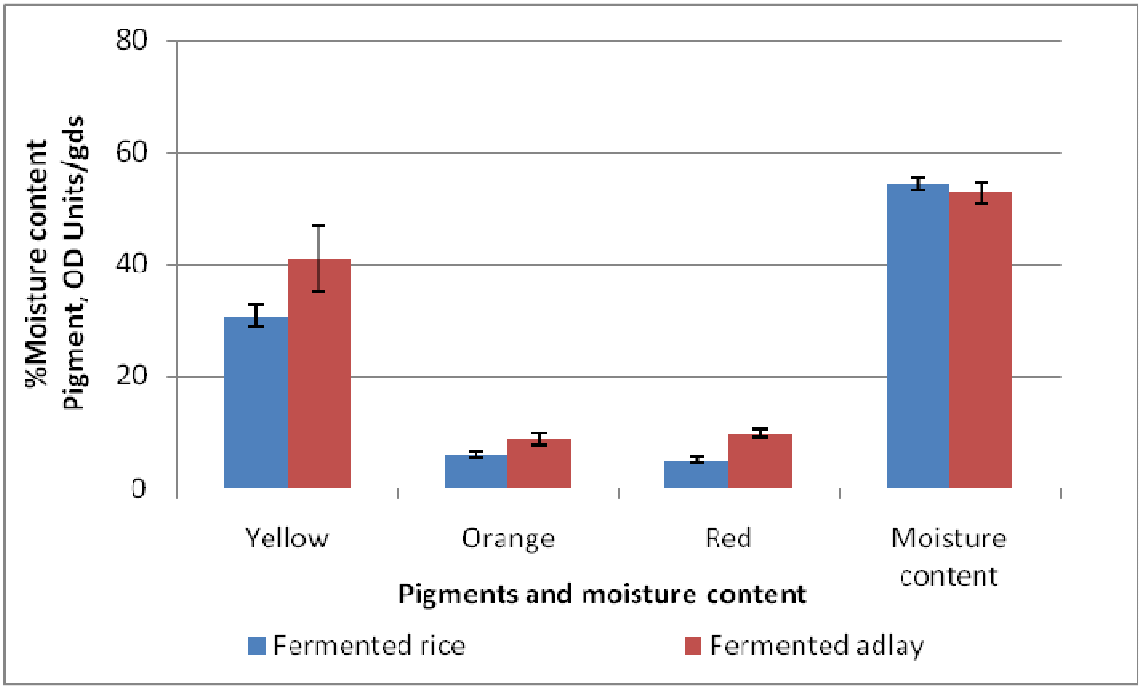
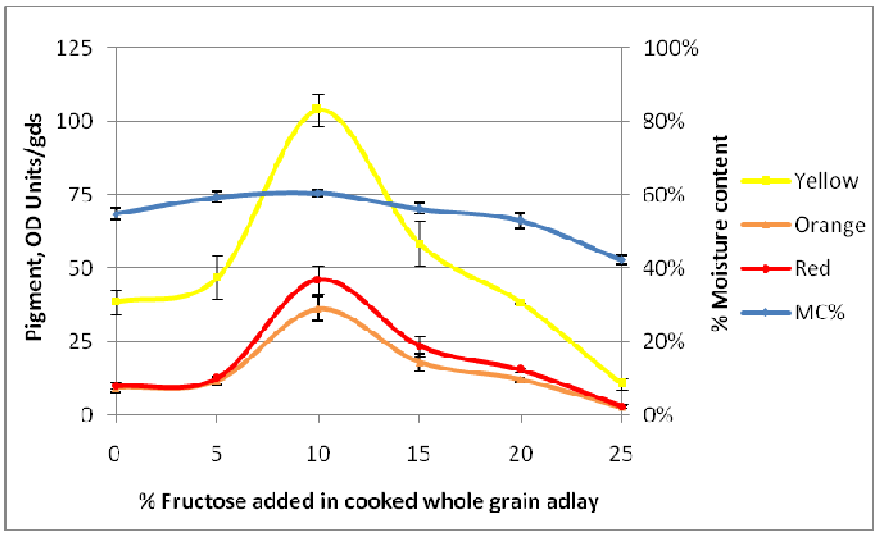


Figure 5.2 *Monascus* fermented rice and *Monascus* fermented adlay in three weeks

5.2.2 Effect of nutrient supplements on *Monascus* pigment production

Schmitt and Blanc (2001) reported that *Monascus* grew quite well on both starch and sugars. As sugars are potentially taken up more quickly, sugars were added to the adlay as additional quickly digestible carbon sources. Fructose, glucose and sucrose were used as they are common sugars which *Monascus* can digest easily. Results are shown in Figure 5.3.

(a)



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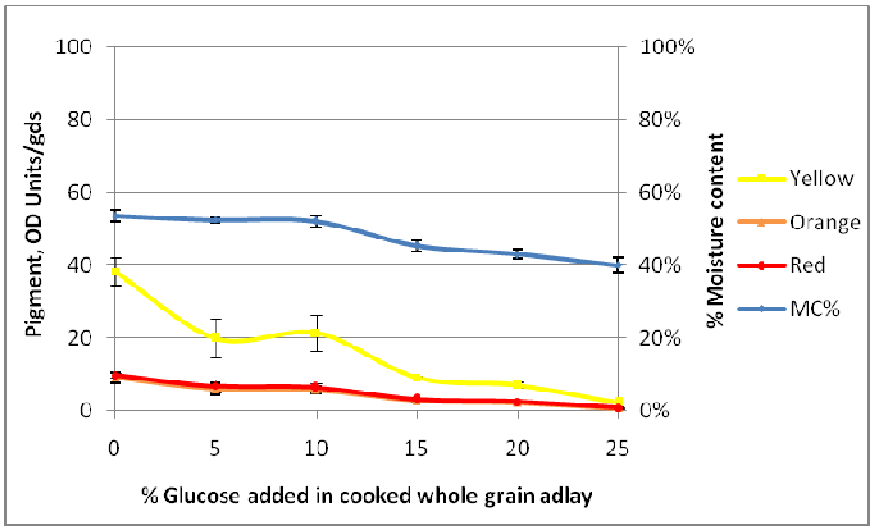

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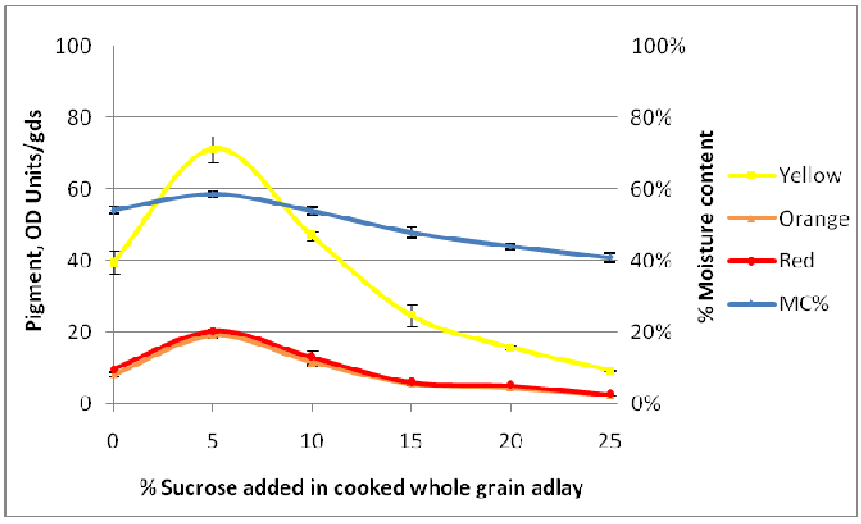


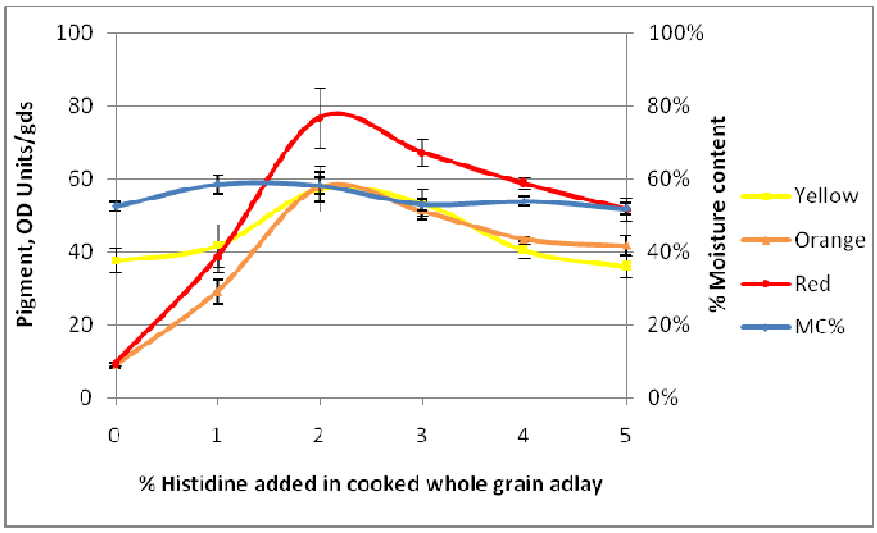
Figure 5.3 Effect of different types of sugar on a three week culture of *Monascus* on adlay. (a) Fructose; (b) Glucose; (c) Sucrose

The type and concentration of sugar clearly affected the pigment yield. The highest yellow, orange and red pigment concentrations were achieved with fructose giving 103.8 ± 5.4, 36.1 ± 4.2 and 46.1 ± 5.1 units per gram, respectively, when 10% sugar was used by total weight. The addition of glucose had a negative effect on pigment production. The addition of sucrose gave maximum pigment concentrations at 5% sugar of 71.2 ± 3.6, 19.1 ± 0.9 and 20.3 ± 0.5 units per gram of yellow, orange and red pigments, respectively. When more than 25% sugar was added, pigment production decreased. When comparing fructose, glucose and sucrose, fructose was found to be the best additive for enhancing pigment production in adlay. These findings are similar to those of Omamor *et al.* (2008), who reported that *Monascus ruber* can grow better in submerged culture on fructose than on glucose or sucrose. Concentrations of over 15% sugar in adlay decreased the moisture content due to the added sugar increasing the percentage of the total solids in the material. Therefore, addition of sugar to adlay had an effect on the water content, and also affected amount of free water / water activity) in the material.

Subhasree *et al.*, 2011 studied the effect of carbon and nitrogen sources to stimulate *Monascus* pigment production on jackfruit seeds. The red pigment yield on jackfruit seed when fructose or starch were added was better than the red pigment yield on jackfruit seed when lactose or mannitol were added. The red pigment yield on jackfruit seed when yeast extract or peptone were added was better than the red pigment yield on jackfruit seed when ammonium nitrate or ammonium sulfate were added.

Chemical supplements such as histidine, monosodium glutamate (MSG) and nitrate were reported as being the best for boosting pigment yield when added to a submerged fermentation of *Monascus* on glucose. Schmitt and Blanc (2001) compared red pigment productivity in glucose with added histidine, monosodium glutamate (MSG) and Soya peptone and found that histidine gave the highest pigment production. However, there is no information about the use of chemical supplements in *Monascus* adlay (adlay angkak).

(a)



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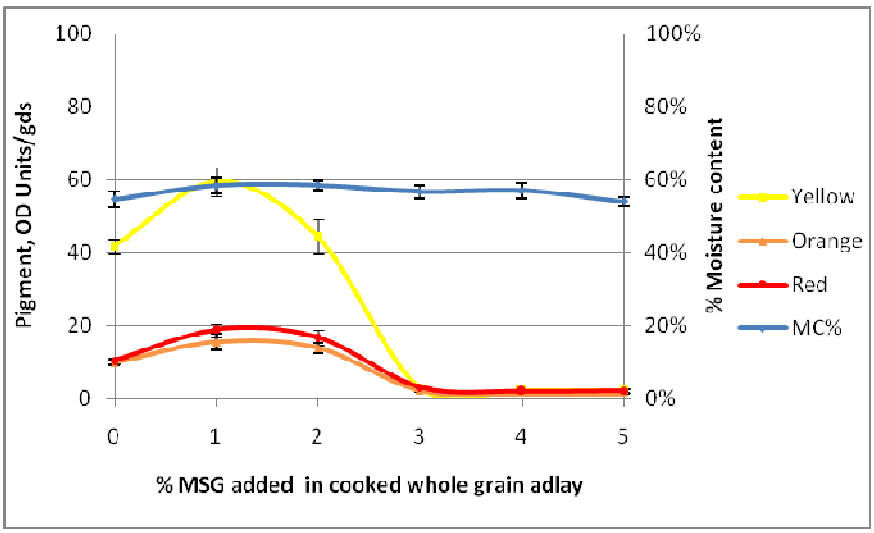

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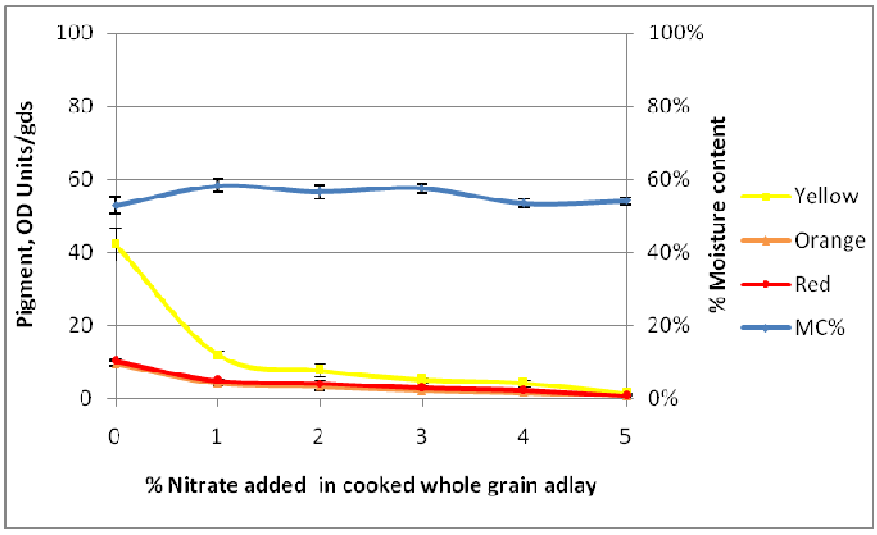


Figure 5.4 Effect of different types of nitrogen sources on a three week culture of *Monascus* on adlay. (a) Histidine; (b) MSG; (c) Sodium nitrate

When *Monascus* was grown in a solid state culture on adlay with different nitrogen sources, it was found that the type and concentration of nitrogen affected the pigment yield (see Figure 5.4). *Monascus* always produced more yellow than orange and red pigments. The addition of histidine had a greater effect on red and orange pigment production than on yellow pigment production. With histidine the highest concentrations of pigments (57.3 ± 6.2, 57.7 ± 4.1 and 76.9 ± 8.1 units per gram of yellow, orange and red pigment, respectively) were obtained when 2% histidine was added. The addition of MSG gave a less high pigment concentration than histidine, but higher than sodium nitrate. The highest pigment production on MSG was obtained at an MSG concentration of 1%, and gave 59.6 ± 4.0, 15.6 ± 2.2 and 18.9 ± 1.9 units per gram of yellow, orange and red, respectively. The addition of sodium nitrate to adlay had a negative effect on pigment production compared with the control. The addition of the different nitrogen sources to adlay had little effect on the water content. In the comparison between histidine, monosodium glutamate (MSG) and sodium nitrate, histidine gave the best pigment production.

In general, the moisture content in adlay had a strong effect on the pigment yield. The change in the moisture content in adlay was very small (about ± 2-3% after three weeks) (see Figure 5.5). Pigment production was quite low at moisture contents below 40% because the availability of water was not enough. Pigment production was maximal at a moisture content of about 60%. At this level the yellow, orange and red pigment concentrations were 49.5 ± 4.8, 13.4 ± 0.7 and 14.7 ± 1.2 units per gram, respectively. At a moisture level of 80% and above oxygen transfer limitation occurred because a water layer formed on the adlay surface, filling the voids between the grains. Pigment production therefore only occurred on the top surface.

The optimum moisture content found in adlay was comparable to that in other systems. Schmitt and Blanc (2001) reported that the optimal moisture content for pigment production by *Monascus* on rice was about 56%. Lower moisture contents (< 32%) led to a large decrease in pigment formation. Babitha *et al*. (2007) reported that 50% moisture in jackfruit gave a higher pigment concentration than 60% moisture. No pigment was produced at a moisture content of less than 45%.

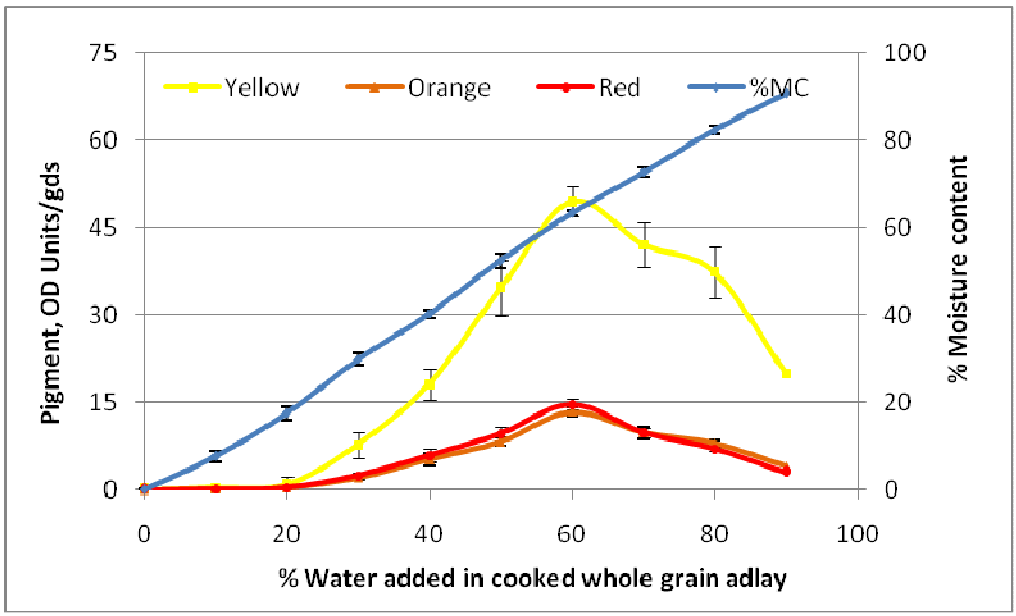


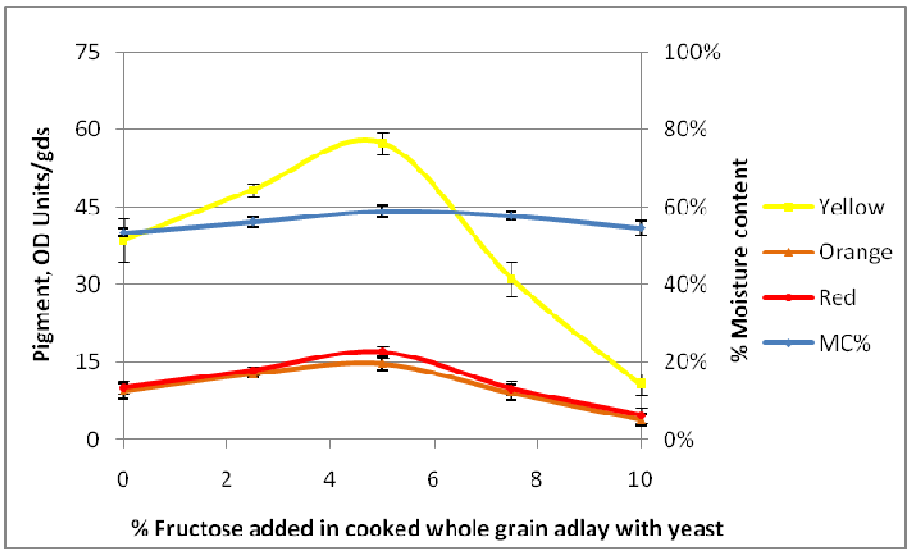
Figure 5.5 Effect of water content on a three week culture of *Monascus* on adlay

5.2.3. Co-culture of *Monascus* and yeast.

The use of elicitors can enhance pigment production during submerged culture and on agar. Enzymes produced by co-cultured microorganisms can also enhance the fermentation process. Different sugar types can affect metabolite production by the co-cultured microorgnaimsis, including enzyme production, and therefore affect the effectiviness of a co-culture. To investigate this further, Monascus was co-cultured with yeast on different types of sugar. Figure 5.6 shows the results. The sugar concentration was only varied between 0 and 10% because in the previous experiments sugar concentration higher than 10% gave reduced yields.

Co-culture of *Monascus* with yeast only slightly increased pigment yield. Co-culture of *Monascus* with yeast gave a maximum pigment yield at 2.5 to 5% of sugar. When fructose was added, the highest pigment yield was achieved with 5% sugar by total weight, giving yellow, orange and red pigment yields of 57.3 ± 2.2, 14.6 ± 1.2 and 17.0 ± 1.2 units per gram, respectively. 2.5% added glucose gave maximum pigment yields of 61.7 ± 4.8, 16.1 ± 0.6 and 17.3 ± 1.1 units per gram of yellow, orange and red pigments, respectively. The addition of 2.5% sucrose to adlay gave a maximum pigment concentration at 61.8 ± 9.2, 16.1 ± 2.4 and 17.6 ± 2.7 units per gram of yellow, orange and red pigments, respectively. The addition of the different sugars to adlay had little effect on the water content.

(a)



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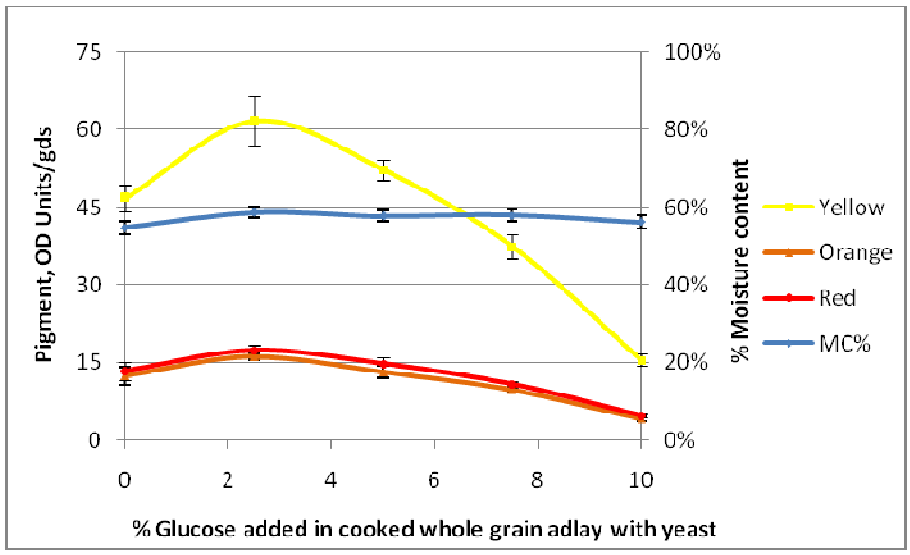

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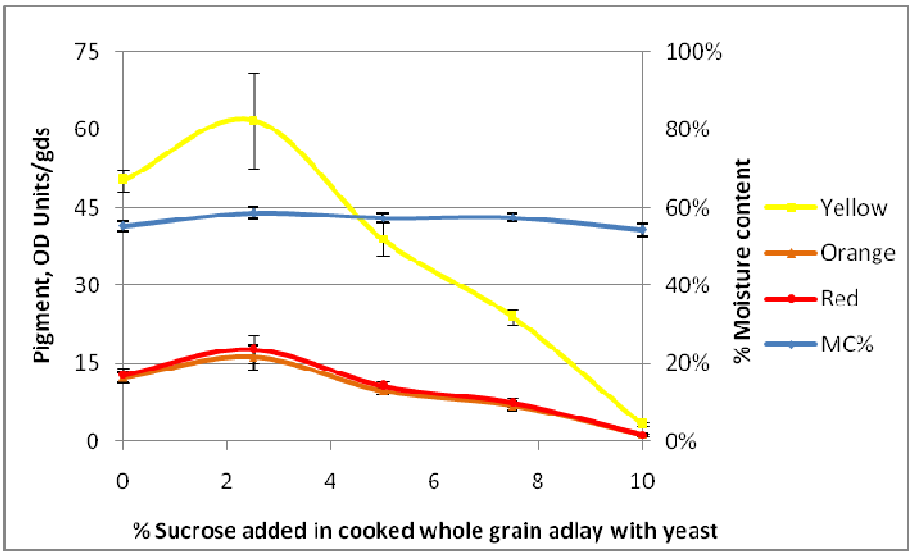


Figure 5.6 Effect of different types of sugar on a co-culture of *Monascus* and yeast on adlay over three weeks. (a) Fructose; (b) Glucose; (c) Sucrose

Shin *et al.* (1998) reported that *Monascus*, when co-cultured with *Saccharomyces cerevisiae* or *Aspergillus oryzae* on agar with sucrose medium, showed enhanced pigment production within seven days. They showed that the addition of some hydrolytic enzymes in sucrose solution affected the morphology of *Monascus*. Furthermore, they proved that the production of amylase, cellulose, protease and chitinase by the yeast and *Aspergillus spp.* played a role in increasing the production of *Monascus* pigment.

The reason for the reduced pigment production may be the fact that yeast growth is promoted at high sugar concentrations. As the sugar concentration increases, it can be expected that the yeast starts to compete with *Monascus* for resources and also produce more waste residues. These effects may have decreased pigment yields

5.3. Conclusions

The period of fermentation of *Monascus* culture on adlay was for four weeks. Pigment production by *Monascus* on whole grains of polished adlay was higher than that on polished rice.

The addition of sugar (fructose, sucrose and glucose) to adlay increased pigment production. The addition of 10% fructose gave the highest pigment yields. Histidine was the best nitrogen source, better than MSG or nitrate, giving a maximum yield of coloured pigment at 2% histidine by total weight. The addition of nitrate actually reduced yield. The addition of histidine increased the production of red and orange pigment more than that of yellow pigment. The water content of *Monascus* adlay strongly affected pigment production. Pigment production was considerably lower when the water content was lower than 40% or greater than 80%. The optimum moisture content was 60%.

The co-culture of *Monascus* with yeast only slightly increased the pigment yield. When sugars were also added, the pigment yield decreased significantly. Co-culture of *Monascus* with yeast can therefore not be recommended for increasing pigment yield.

CHAPTER 6

OPTIMISATION OF THE PRODUCTION OF PIGMENT BY *MONASCUS* ON PUFFED ADLAY DURING A FIXED-TRAY FERMENTATION

6.1. Introduction

In China, the solid state fermentation of *Monascus* has been used to produce food and health remedies for over 1000 years (Feng *et al.*, 2012, Shi and Pan, 2011 and Wang and Lin 2007). *Monascus* grown on rice, called Hong Qu, Hon-chi, Anka or Angkak, has been – and is still - used as a natural pigment in fish (surimi and other marine products), Chinese cheese, red wine, tomato ketchup and meat products (sausages and hams).

Pigment production by *Monascus* nowadays is done by both solid state and submerged fermentation. 90% of the production is nowadays done in submerged culture, mainly because it is less labour intensive and more easily controlled. However, *Monascus* culture by solid state fermentation on whole grains can give higher pigment production rates and at a cheaper cost than in submerged culture. Furthermore, the extraction of pigment from solid state culture is more efficient due to the fact that the product to be isolated is present in a concentrated form at the start of the extraction process (Carvalho *et al.*, 2006). Moreover, waste water production is lower than for liquid fermentation (Couto and Sanromán, 2006). On the negative side, solid state culture is almost always slower than liquid fermentation due to the slow conversion of the material.

Slow conversion of a substrate is a common problem in food production, and many methods have been developed by which a material can be pre-treated before conversion. Common methods of pre-treatments of grains and related materials such as nuts are coating, blanching, soaking and puffing treatments.

Coating involves the application of a thin layer of material over the surface of the particles. A typical application of a coating is as a barrier against moisture, oxygen and solute movement to increase the shelf life (Baldwin and Wood, 2006, Bourtoom, 2008, Gulbert, 1987, Han *et al.*, 2008, Lee *et al.*, 2002 and Mat'e *et al.*, 1996). The coating has to be edible if the product is to be used as food.

The purpose of blanching is to destroy enzymatic activity, modify texture, preserve colour and remove trapped air. Blanching is used in the processing of many cereals, fruit and vegetables (Kaack, 1994, Reyes-De-Corcuera *et al.*, 2004 and Wambura and Yang, 2010). Starch in a material may become gelatinized when blanching at high temperatures. Soaking is a method that is used with hard grains to soften texture and improve the quality of cooking (Rehman *et al.*, 2001 and Rehman 2007).

The puffing process is a process that expands the food material using a pressure-driven process. It based on a sudden application of different pressures under heat so that the water in the grain is vaporized *in situ,* thereby expanding and drying the product simultaneously. Puffed cereals are ready-to-eat cereal products which humans can consume without requiring further cooking and are commonly used as breakfast foods or as ingredients in snacks. Commonly puffed cereals are maize, wheat, oats and rice. Puffed materials are appreciated mainly for their lightness and crispness in relation to their cellular structure (Mariotti *et al.*, 2006 and Hoke *et al.*, 2007).

Coating adlay is not really suitable as a pre-treatment to enhance the fermentation rate of the adlay. It is not clear how a coating on the surface could enhance the conversion rate inside the particles. It is more likely to reduce the conversion rate as it gives an additional barrier to the diffusion of oxygen. Apart from this, physical modification of products generally has a higher customer acceptance than chemical treatment such as coating.

Blanching and soaking are conventional treatment methods. Although it has been shown that the pigment yield during the culture of *Monascus* on corn cob increased when soaking time is over 48 hours (Velmurugan *et al.*, 2011), the effects of blanching and soaking will most likely be overshadowed by the cooking step that is done before the fermentation of the adlay. Puffing however is likely to have a large

effect as a pre-treatment because it increases the porosity of the starting material. The many pores of puffed grains can absorb a large amount of water Mariotti *et al.* (2006) reported that puffed grains could take up over 300% of their weight in the first 30 seconds while raw grains did not reach these values even after 20 hours and had a water uptake of only 100%. Penetration of the fungus into the grain during fermentation can be expected to be faster than in whole grains. The starch will be more accessible. The penetration of oxygen is also potentially faster. The conversion of the grain material into pigments should therefore be much quicker in puffed grains than in common grain. It was therefore decided to look at puffed adlay as a potential new substrate for the production of adlay angkak.

6.2. Results and discussion

6.2.1. A comparison of pigment production on puffed and non-puffed adlay.

The culture of *Monascus* was prepared in a polycarbonate plastic box (see 3.4.1.3 in Chapter 3). Figure 6.1 shows the pigment yield after the culture of *Monascus* on whole grain adlay and puffed adlay was fermented for three weeks. The concentrations of red and orange pigments produced by the culture on puffed adlay were slightly higher than on the whole grain adlay. The concentrations of yellow pigment were similar. Total pigment production on the puffed adlay was slightly higher than that on whole grain adlay.

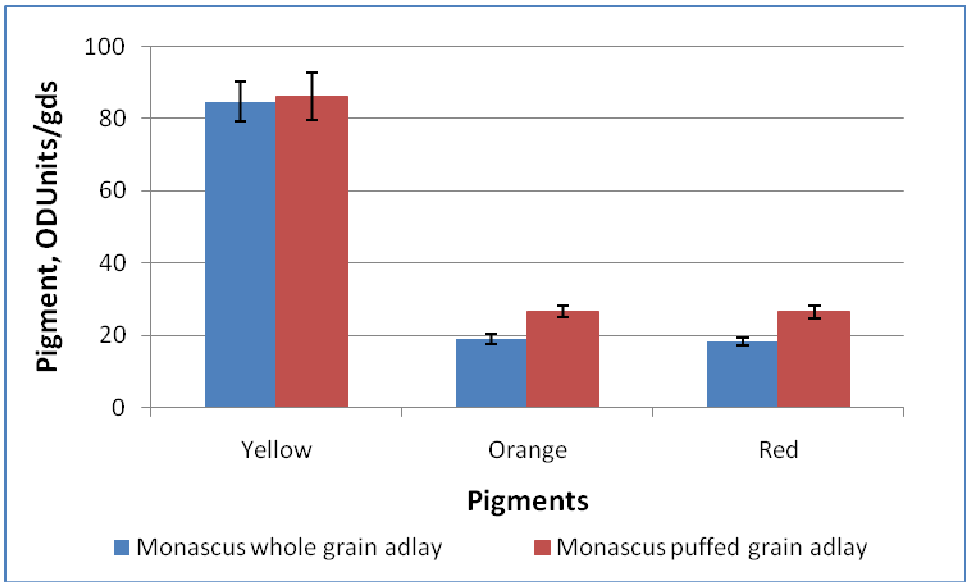


Figure 6.1 Comparison of pigment production by *Monascus* on whole grain adlay and puffed adlay

Figure 6.2 and 6.3 shows the progression of pigment production during the culture of *Monascus* on whole grain adlay and puffed adlay in universal bottles (see 3.4.1.4 in Chapter 3). Pigment concentration levels became stationary during the culture on the whole grain adlay. It took around 15 days on the whole grain adlay after inoculation whereas on puffed adlay it was just 12 days. The rate of pigment production on the puffed adlay was therefore faster than that on the whole grain adlay and total pigment production on the puffed adlay was also higher.

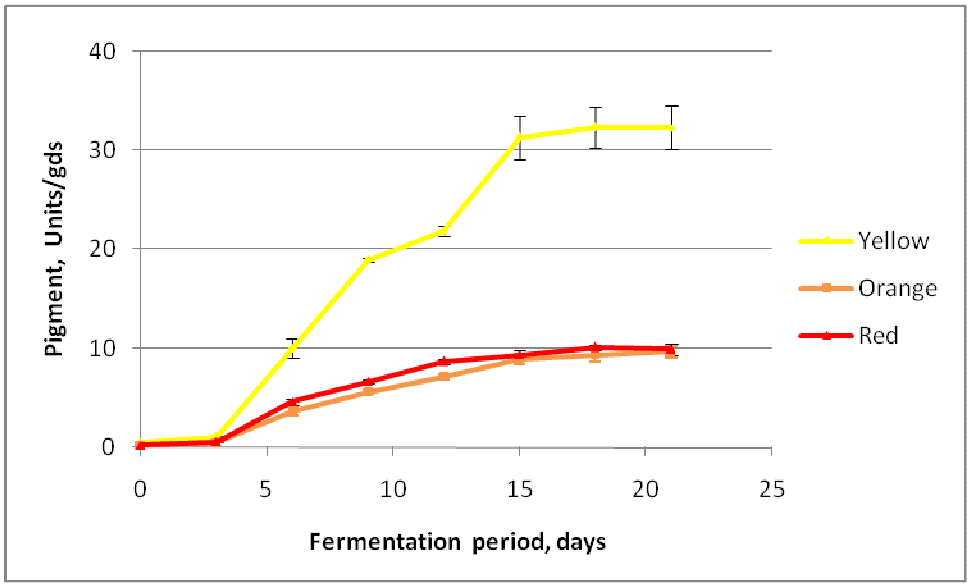


Figure 6.2 Pigment production rate on single layer of whole grain adlay

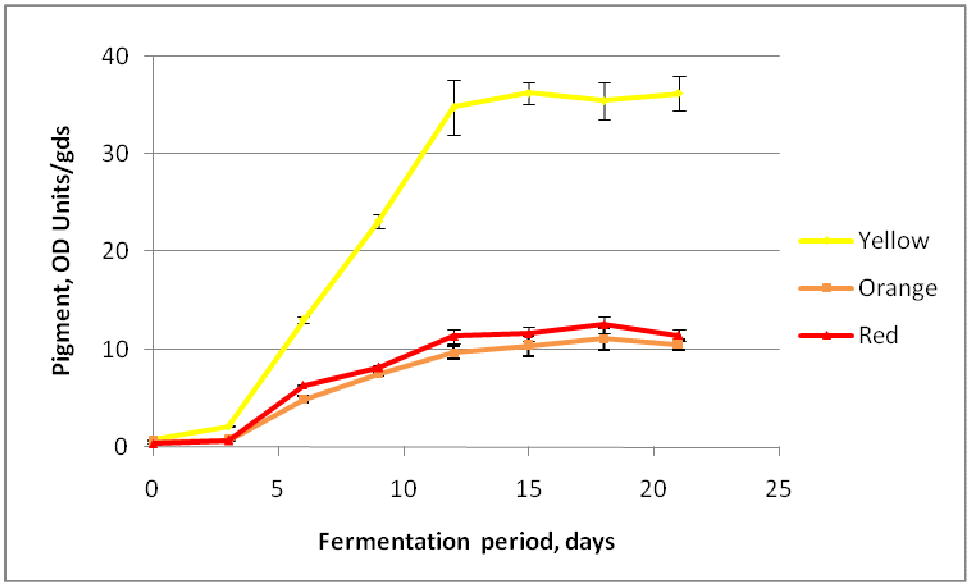


Figure 6.3 Pigment production rate on single layer of puffed grain adlay

6.2.2. Changes in the particle size and shape of whole grain adlay and puffed adlay after cooking and fermentation

In the previous experiments, it was observed that considerable changes in grain size occurred when the (puffed) adlay grains were cooked. To investigate this further, changes in the grain size were measured before and after cooking, and after fermentation (see 3.7.7 in Chapter 3). The diameter of raw grain of common whole grain was found to be 4.9 ± 0.23 mm, whilst that of the puffed adlay was 8.5 ± 0.33 mm. The average diameter of puffed grains was therefore 1.7 times larger than that of whole grains and the average volume of puffed grains was around five times bigger than that of whole grains. The shape of puffed adlay grains was also more round compared to whole unpuffed grains of adlay (see Figure 6.4). The fact that the particle size of adlay grains after puffing was significantly bigger in comparison to raw grains means that on the inside puffed adlay should be much more porous. This large porosity inside the grain could have benefits for the culture of *Monascus* on adlay.

The change in particle size could also be observed when measuring the density of a bed of grains. The average bulk density of common adlay grain was found to be around 774 ± 14 grams per litre whereas for puffed adlay it was just 78.6 ± 3.7 grams per litre. The degree of expansion from raw to puffed material was therefore about ten.

Grains of both types were mixed with water and cooked in an autoclave. The size of the grains changed during the cooking process. The major axis of grains of normal adlay increased from 4.94 ± 0.23 mm to 5.76 ± 0.27 mm. as the particles took up water during the cooking process. The average size of puffed adlay grains, however, decreased from 8.57 ± 0.33 mm to 5.98 ± 0.27 mm. The smaller size of the puffed adlay particles after cooking indicates strongly that the cooking process significantly reduces the porosity of the puffed grains.

Grains of both types were then fermented in a single layer in universal bottles. After fermentation, the fermented grains of both types were found to be similar in size to the grains before inoculation.

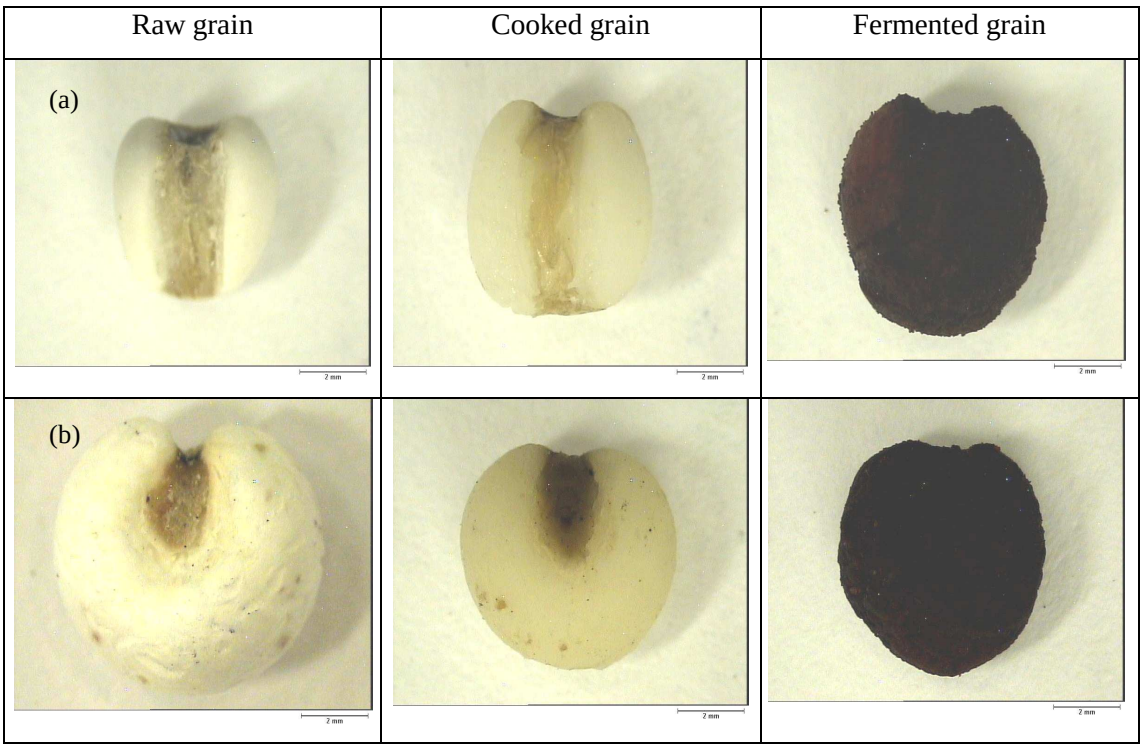


Figure 6.4 Images of grains taken with a Leica microscope
(a) Common whole grain adlay (b) Puffed adlay

6.2.3 Comparison of the rate of penetration of pigment production inside kernels of non-puffed and puffed adlay.

In chapter 4, it was shown that pigment production during *Monascus* fermentation progressed from the top of a bed of adlay to deeper layers, and was limited by the diffusion of oxygen. The experiments did not provide information about the rate of penetration of the fungus into individual grains or the production of pigment inside the grains. To study this, the progression of pigment penetration over time into individual whole adlay grains was studied. Culture was done in a single layer at the bottom of a universal bottle in order to prevent oxygen limitation at the surface of the particles (see 3.4.1.4 in Chapter 3).

Figure 6.5 gives images showing the typical progression of pigment production within grains of non-puffed adlay and puffed grains of adlay during the three weeks of study.

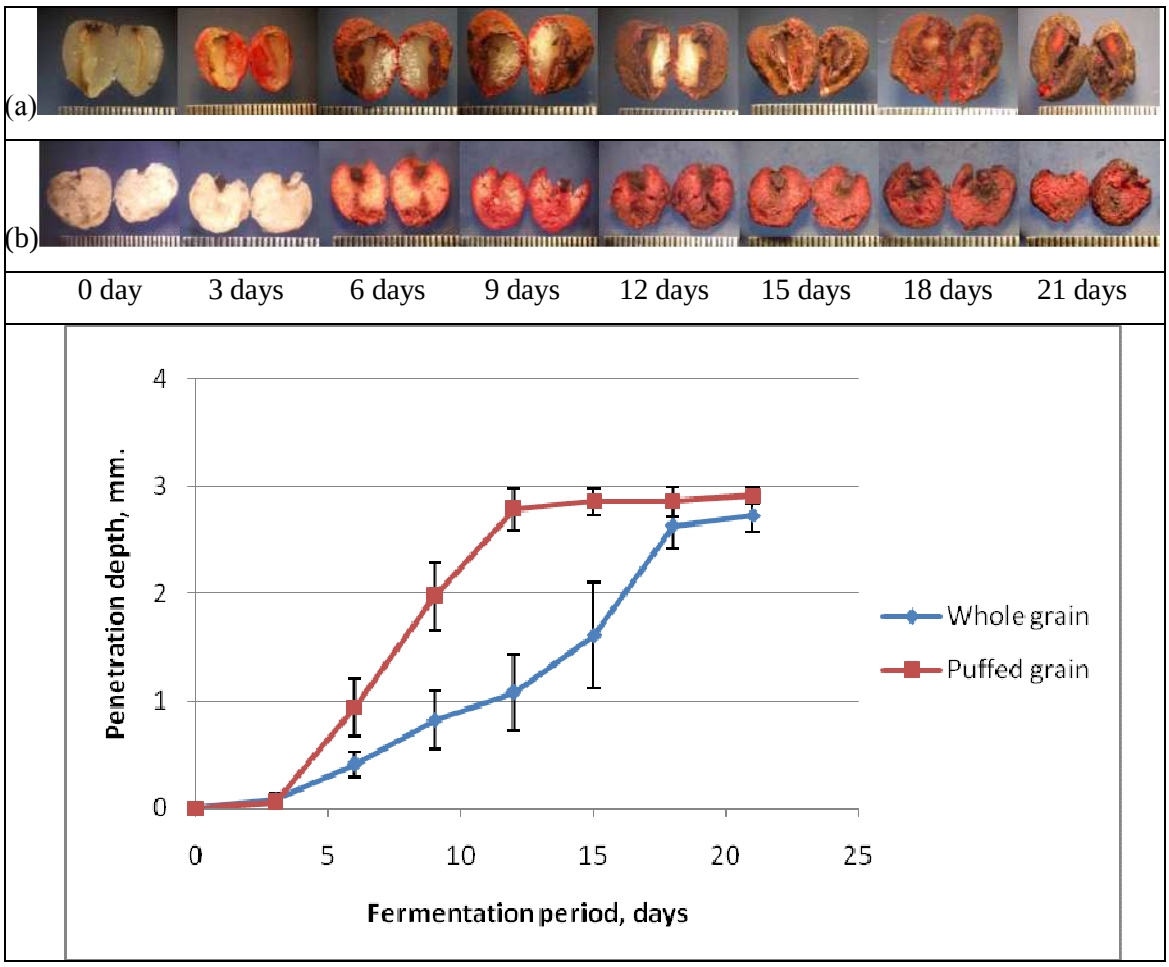


Figure 6.5 Pigment penetration inside kernel

(a) Common whole grain adlay (b) Puffed grain

After three days, red pigment could be seen on parts on the outside of both types of grains but little pigment could be found inside the grains. After six days of fermentation, red pigment had fully covered the outside of the grains but red pigment could only be found in some parts inside the grains. After six days, pigment production could clearly be seen to progressing inside the grains. After 12 days, pigment production had taken place throughout the particles of cooked puffed adlay. Only after 15 days was a similar level of pigment penetration reached in cooked non-puffed adlay, even though some non-converted adlay could still be observed.

6.2.4 Investigation of the structure of puffed and non-puffed adlay using a scanning electron microscope (SEM)

To investigate the issue of porosity further, the structure of the inside of the kernels of whole and puffed grain were studied before and after cooking and after fermentation using a scanning electron microscope (see 3.7.7 in Chapter 3). Images from the scanning electron microscope, shown in Figure 6.6, indicated that grains of raw common adlay had a smooth surface, whilst the inside of common adlay was relatively compact and dense with few or no pores, and was full of starch granules (Figure 6.6). This is similar to that of other cereals. For example, Jane *et al.* (1994) reported that, at high magnification, grains of wheat, emmer wheat, rye and barley can be seen to be composed of granules of different sizes: 2-3 µm for the small granules and 20 – 35 µm for the large ones.

The surface of puffed grain still was rougher in comparison to that of normal unpuffed adlay (see Figure 6.6a and 6.7a). The inside of the puffed grains was very porous with pores of around 15 - 40 µm separated by a thin wall. These findings are similar to those reported by Mariotti *et al.* (2006), who studied the effect of puffing on the structure of grains of wheat, rye, rice, emmer wheat, barley and buck wheat. They showed that the puffing treatment significantly changed the ultrastructure of the grains. Before the puffing process had an internal structure that was compact and relatively homogeneous, and after puffing grains had a porous structure with a wide distribution in pores sizes. Mariotti *et al.* (2006) also reported there was a large distribution in sizes of the pores. The size of the pores increased from the centre of the kernel outwards. They varied from 2 µm to up to 600 µm and depended on the position in the grain and the cereal type. Mariotti *et al.* (2006) also reported that the pressure changes at the high temperatures produced during the puffing procedure can damage up to 80% of the starch.

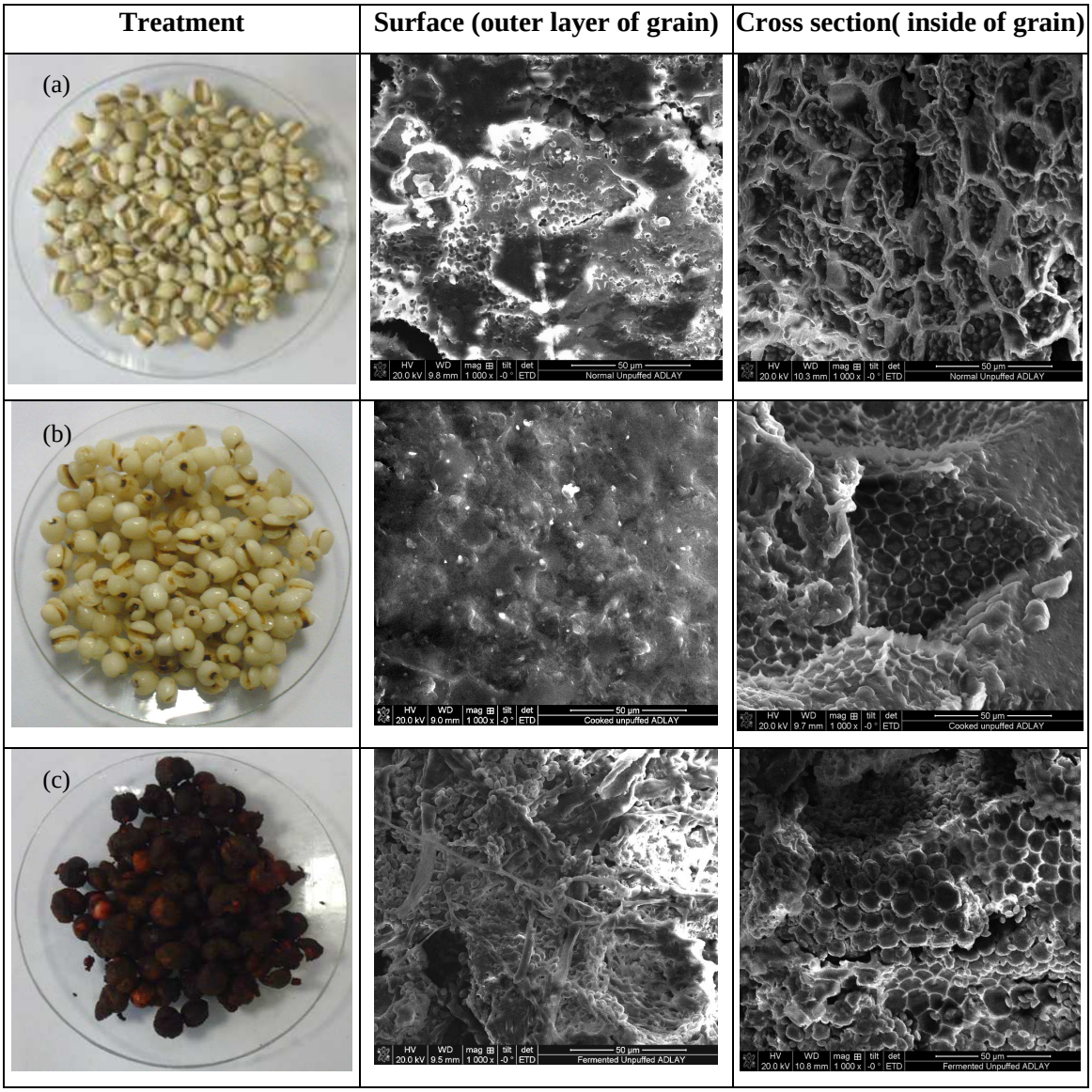


Figure 6.6 Common whole grain adlay and SEM micrographs thereof.
(a) Raw grain (b) Cooked grain (c) Fermented grain

Figure 6.6b and 6.7b show SEM images of cooked non-puffed and puffed adlay. After cooking, the normal adlay grain had absorbed water and was larger. It still had a smooth surface and was still compact with starch granules inside the kernel. The outside surface of cooked puffed adlay was still rougher. The size of the cavities inside the puffed kernel had decreased, and many had fused but at the same time many big holes could be found of around 10 - 20 µm inside the kernels.

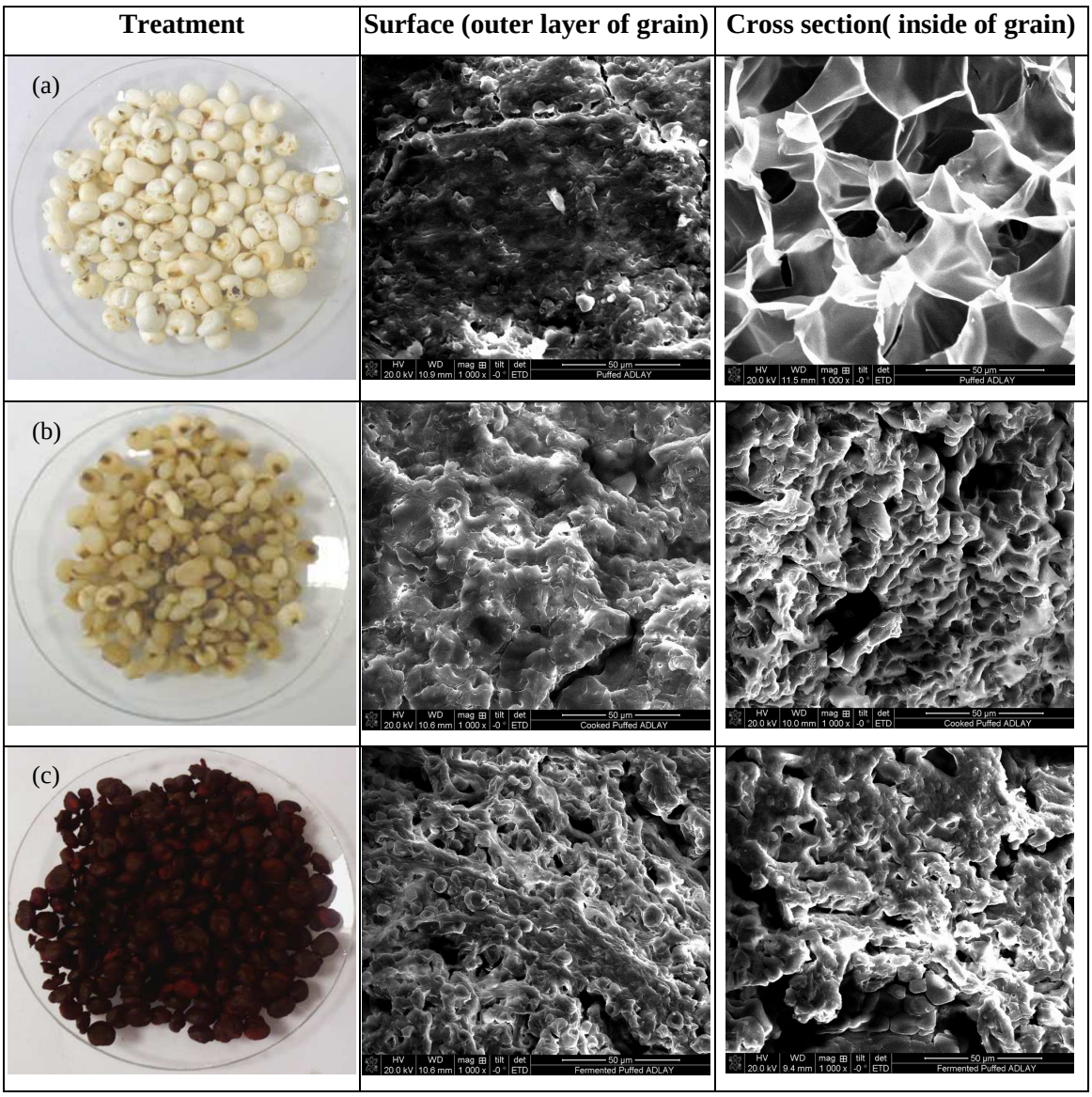


Figure 6.7 Puffed adlay and SEM micrographs thereof.
(a) Raw grain (b) Cooked grain (c) Fermented grain

Figure 6.6c and 6.7c show SEM images of the fermented products. There were only a few changes in the structure of fermented grains when compared with the structure of cooked grains. The outside surface of cooked whole grains still had a smooth surface, though its surface was covered with red fungal mycelium. The inside of the kernel was covered with the red pigment granules and had somewhat compacted, giving the material less porosity. The surface of fermented puffed adlay was still rough but was covered with red fungal mycelium. The inside of fermented puffed adlay still had many macropores and micropores and was covered with red pigment.

6.2.5. Optimisation of the culture of *Monascus* on puffed adlay

In the next experiment, the influence of the addition of fructose, histidine and water on a culture of *Monascus* on puffed adlay was studied.

The influence of the addition of fructose, histidine and water on pigment yields and glucosamine to the culture of *Monascus* on puffed adlay was investigated using a response surface method and, in particular, a central composite design, as described by Montgomery (1997). A 2^3 factorial experiment was performed with six axial runs and three replicates in central points. Similar methods were used by Chang *et al.* (2002) and Silveira *et al.* (2007).

The culture was done in polycarbonate plastic boxes which were used as tray bioreactors (see 3.4.1.3 in Chapter 3). In the previous chapter, it was found that a layer of adlay of more than 4 cm reduced *Monascus* pigment yield, so the thickness of adlay layer in the trays was kept less than 2 cm. Puffed adlay was mixed with nutrient sources in the trays according to the schedule shown in Table 6.1 and sterilized in an autoclave. The medium was inoculated and kept in an incubator at 30°C for 21 days. After that, the pigment and the glucosamine concentration were determined. Design Expert version 6.0.2 was used to find the optimal conditions for pigment production. The response surface quadratic model of the program Statistica for Windows version 4.3 was used to make the graphs.

Table 6.1 Nutrient formula for additives to 100 g puffed adlay in fixed-tray bioreactors

Treatment	**Water grams**	**Fructose grams**	**Histidine grams**	**Treatment**	**Water grams**	**Fructose grams**	**Histidine grams**
1	230.00	20.00	4.00	10	128.92	14.05	2.81
2	230.00	20.00	4.00	11	331.08	14.05	2.81
3	230.00	20.00	4.00	12	60.00	20.00	4.00
4	128.92	14.05	5.19	13	400.00	20.00	4.00
5	331.08	14.05	5.19	14	230.00	10.00	4.00
6	128.92	25.95	5.19	15	230.00	30.00	4.00
7	331.08	25.95	5.19	16	230.00	20.00	2.00
8	128.92	25.95	2.81	17	230.00	20.00	6.00
9	331.08	25.95	2.81				

Data on pigment and glucosamine production obtained are shown in Table 6.2.

Table 6.2 Effect of nutrient formula on *Monascus* puffed adlay in a fixed-tray bioreactor

Treatment	Glucosamine mg/gds	Average optical density, Units/gds		
		Yellow	Orange	Red
1	93.68	70.35	72.08	94.25
2	111.83	82.91	77.41	110.96
3	100.18	77.23	70.99	101.74
4	76.85	28.18	33.88	39.90
5	80.93	79.43	86.04	94.63
6	70.03	15.62	11.10	12.97
7	92.95	36.27	24.55	29.86
8	88.43	55.90	50.33	71.19
9	80.95	22.67	19.11	20.60
10	94.40	65.44	38.22	52.53
11	75.88	43.46	33.49	40.03
12	86.35	74.29	62.13	81.09
13	82.85	40.51	30.10	37.79
14	88.65	41.57	31.44	41.11
15	98.53	41.84	30.70	40.94
16	69.60	49.16	20.08	23.32
17	89.68	34.27	23.58	29.47

The additives had clearly affected both glucosamine and pigment production. The histidine concentration appeared to have affected pigment production more than fructose and water content. However, the relationship between the concentration of the additives and the glucosamine/pigment concentration was complex. A mathematical model was therefore needed to describe that relationship.

The amounts of water (g) , fructose (g) and histidine (g) added to adlay (100 g dry solid matter (gds)) were set as variables X, Y and Z respectively and were fitted as polynomial functions in the pigment concentrations. The highest order of the polynomial model was selected from the sequential model sum of squares (see Table 6.3). The predicted equation was significant and fitted with the statistical model if the probability was more than 95% ($p < 0.05$). Coefficient of determination could be found

between the concentration of the additives and the pigment concentration, with r-square values for all pigments of more than 0.85.

Table 6.3 Sequential Model Sum of Squares in yellow, orange and red pigment

Response: Yellow						
Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	Remark
Mean	43415.52	1	43415.52			
Linear	369.18	3	123.06	0.24	0.8679	
2FI	2991.97	3	997.32	2.69	0.1033	
Quadratic	2880.79	3	960.26	8.07	0.0113	Suggested
Cubic	744.53	4	186.13	6.30	0.0812	Aliased
Residual	88.63	3	29.54			
Total	50490.62	17	2970.04			
Response: Orange						
Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	Remark
Mean	28430.88	1	28430.88			
Linear	435.98	3	145.33	0.28	0.8384	
2FI	1392.29	3	464.10	0.87	0.4887	
Quadratic	4427.59	3	1475.86	11.34	0.0045	Suggested
Cubic	834.59	4	208.65	8.17	0.0579	Aliased
Residual	76.63	3	25.54			
Total	35597.96	17	2094.00			
Response: Red						
Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	Remark
Mean	50045.42	1	50045.42			
Linear	913.05	3	304.35	0.27	0.8481	
2FI	3337.56	3	1112.52	0.97	0.4455	
Quadratic	9399.38	3	3133.13	10.48	0.0056	Suggested
Cubic	1812.65	4	453.16	4.85	0.1126	Aliased
Residual	280.20	3	93.40			
Total	65788.25	17	3869.90			

The equation in terms of actual factors were:

$$\text{Yellow} = -242.8267 - 0.1506X + 21.1761Y + 69.1314Z - 0.0007X^2 - 0.3621Y^2 - 9.0495Z^2 - 0.004XY + 0.1321XZ - 1.5191YZ \qquad R^2 = 0.88$$

$$\text{Orange} = -313.6784 - 0.0085X + 19.4895Y + 102.2069Z - 0.0008X^2 - 0.3986Y^2 - 12.274Z^2 + 0.0016XY + 0.0848XZ - 1.1745YZ \qquad R^2 = 0.87$$

$$\text{Red} = -453.685 + 0.0086X + 28.2437Y + 144.1567Z - 0.0013X^2 - 0.5712Y^2 - 17.9369Z^2 + 0.0001XY + 0.14XZ - 1.6327YZ \qquad R^2 = 0.87$$

Where X is the amount of water added (g/100 g adlay)

Y is the amount of fructose added (g/100 g adlay)

Z is the amount of histidine added (g/100 g adlay)

Attempts to do the same with the glucosamine concentration were less successful. The glucosamine data from experiments fell into a narrow range of values. There was too much noise in the data to obtain a good fit. Therefore, predicted equation of glucosamine was non-significant. It was therefore decided to analyse only the pigment data further.

Based on the results obtained, it can be predicted that optimal pigment production on puffed adlay can be expected when 20, 4 and 200 gram are added per 100 gram dry solid matter of fructose, histidine and water, respectively. The predicted concentrations of yellow, orange and red pigments are 78, 74 and 103 units per gram dry solid, respectively.

More details of the relation between the predicted pigment concentrations and the concentration of the nutrient source are shown in Figures 6.8 and 6.9. In Figure 6.8, the predicted pigment concentrations are shown at different moisture levels when the fructose concentration is 20 g per 100 g dry puffed adlay, and the histidine concentration 4 g per 100 g dry adlay. The predicted data correlate well with experimental data. The predicted concentration of red pigment is higher than that of the orange and the yellow pigments at all moisture levels, with a maximum at 67%. The predicted pigment concentration increases when the water content of adlay is increased. The pigment concentration began to reduce when the level of water content is over 70%. Oostra *et al.* (2000) reported that the water activity equals 1.0 when the water content in oats was over 55%. This means that the vapour pressure of the water in the system is the same as the vapour pressure of pure water at the same temperature. Fermented puffed grain absorbed much more water due it to having a higher porosity.

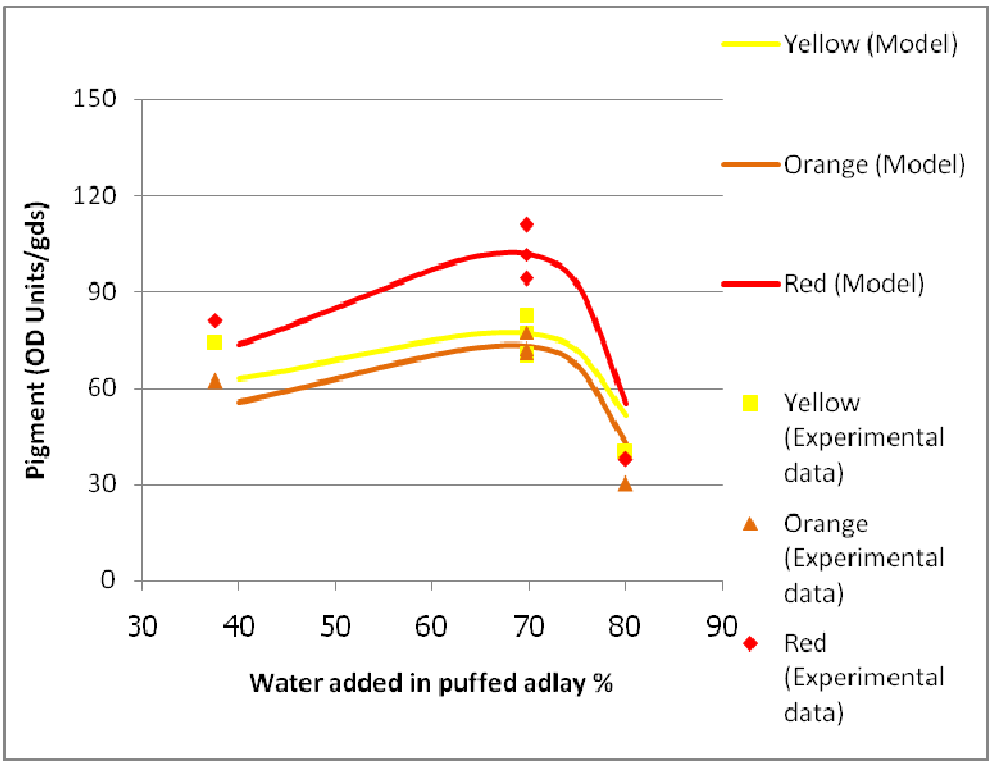


Figure 6.8 Predicted effect of moisture levels on pigment production when water is added to 100 g puffed adlay with fructose 20 g and histidine 4 g

Figure 6.9 shows the predicted pigment concentrations at different fructose and histidine concentrations at a moisture level of 67% in contour and response surface charts. The experimental data are compared with the predicted data in these graphs. The colour dots (▫ , ▪ and ▪) represent the experimental data in yellow, orange and red repectively. The white dots represent data predicted from model. The predicted data correlate well with the experimental data when histidine and frustose concentrations were varied. Less histidine was needed to increase the pigment production than fructose. Therefore, it would be better to add histidine as a nitrogen source than fructose as a carbon source.

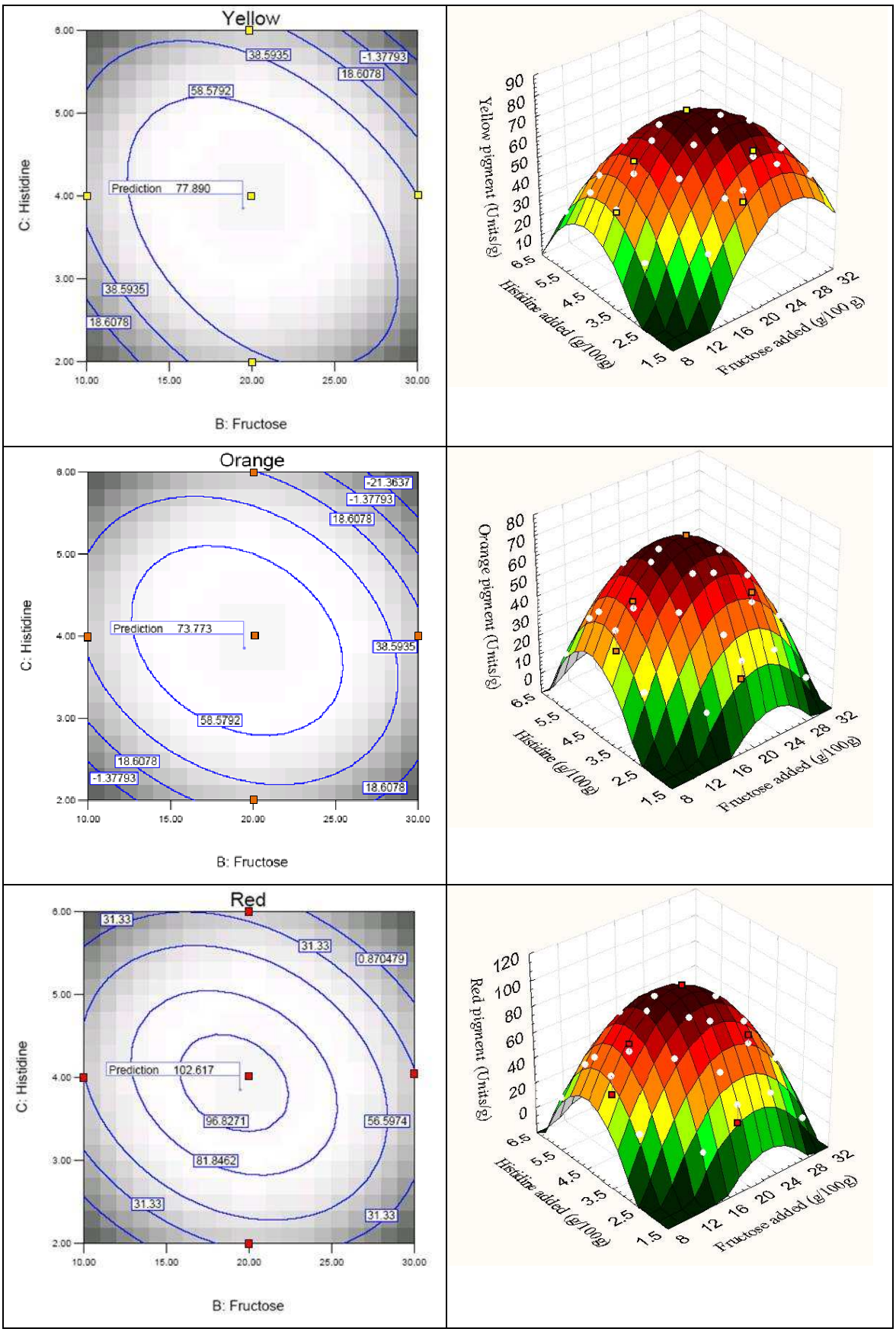


Figure 6.9 Contour and response surface charts showing the predicted effect of the addition of fructose and histidine on pigment production in puffed adlay with a moisture content of 67%

6.3. Conclusions

A comparison of a culture of *Monascus* on whole grain non-puffed adlay and puffed adlay has shown that puffed adlay is superior as a substrate. The conversion of puffed adlay was faster than that of whole grain adlay, and the final concentration of pigment was also higher. The reason for the better performance of puffed adlay as a substrate lay in its higher porosity. Even though the puffed adlay shrunk significantly during the cooking process, the remaining porosity was high enough to increase the rate at which the fungus penetrated the kernel and converted the starch. Using puffed adlay in the production process would make it possible to obtain a relatively homogeneous product more quickly. Variation of the composition of the substrate bed showed pigment production could be enhanced further by adding additional nutrients; optimum production was achieved by adding 200 g water, 20 g fructose and 4 g histidine per 100 g of dry adlay.

CHAPTER 7

PRODUCTION OF *MONASCUS* PIGMENT WITH A ROTATING BIOREACTOR

7.1. Introduction

Solid state fermentation (SSF) is the oldest biotechnological process and has been used for at least 3000 years (Hölker *et al.*, 2004 and Pandey *et al.*, 2008). The recorded history of solid state fermentation during the production of cheese and Koji was described in Asia before the birth of Christ. Solid state fermentation involves the growth of microorganisms on a moist solid substrate in the absence of free flowing water. The water content is quite low and on the surface the microorganism is in direct contact with gaseous oxygen in the air (Augustine *et al.*, 2006 and Raghavarao *et al.*, 2003).

There are many factors which affect biomass and pigment yield during solid state fermentation, such as the availability of nutrients (carbon source, nitrogen source and minerals), and moisture (Jůzlová *et al.*, 1996, Dufossé *et al*., 2005, Babitha *et al.*, 2007, Omamor *et al.*, 2008). Water content in particular greatly affects pigment production. If the water content is too high, voids in the material are filled with water and air is driven out. This creates anaerobiosis. Low moisture content, on the other hand, may affect cell growth, metabolites and the other products. High moisture content in the starting material might also give a highly sticky material after cooking (Keawkaika *et al.*, 2010 and Kainuma, 2006). Commonly used cereal grains such as rice, wheat, barley and rye become sticky after cooking. Stickiness depends on many factors including the variety of grain used, the degree of milling, cooking conditions (water content, temperature, pressure and etc.) (Leelayuthsoontorn *et al.*, 2006 and Mohapatra and Bal, 2006). Stickiness affects oxygen transfer; it may also cause the particles to adhere to each other, which can make further processing difficult. Therefore, moisture content needs to be controlled during fermentation.

Bioreactors used for solid state fermentation can be divided into four types: fixed trays, packed beds, rotating horizontal drums and fluidized bed bioreactors. Tray and packed bed bioreactors have a fixed bed that is not mixed; the distribution of any product produced is therefore non-homogeneous. Rotating horizontal drum and fluidized bed reactors have a moving bed. As a result, production is more homogeneously spread over the bed. It is also easier to remove heat, and mass transfer between the particles and gas phase is better (Couto and Sanromán, 2006, Durand, 2003, Hardin *et al.*, 2002, Mitchell *et al.*, 2000, Pandey, 1991, Raghavarao *et al.*, 2003 and Singhania *et al.*, 2009). Continuous production is possible with a moving bed but not with a fixed bed.

Mitchell *et al.* (2006) reported that *Monascus* pigment and other fungal products such as enzymes are produced at much higher yields in solid state culture than in submerged cultures. Even though many different reactor types can be used for solid state fermentation of *Monascus* (Couto and Sanromán, 2006, Rosenblitt *et al.*, 2000, Said *et al.*, 2010 and Wang and Lin, 2007), commercial solid state culture of *Monascus* culture has, to date, been limited to tray bioreactors. A few applications have been described in which the solid state of *Monascus* is done in packed bed bioreactors (Han and Mudgett, 1992, Rosenlitt *et al.*, 2000 and Said *et al.*, 2010), all at laboratory scale. There is therefore significant scope for further development in bioreactor technology for the solid state culture of *Monascus* for pigment production.

To date, *Monascus* culture has been limited to non-moving beds, but to achieve a homogeneous product that can be produced continuously, it would useful to try a system with a bed in which the substrate can be mixed. The mixing of the substrate will spread the inoculum throughout the bed at the start of the fermentation, ensuring the fermentation starts at the same time everywhere, and will also ensure homogeneous production during the fermentation process. Because of the high particle density and the expected sticky nature of the grains after cooking, a fluidised bed would not be the first choice. A rotating bioreactor however appears quite suitable. Although there have been many studies on rotating solid state cultures, work to date has concentrated on developing processes to increase productivity on solid waste (palm kernel cake, grape pomace and solid sludge waste) (Diaz *et al.*, 2009, Fung and Mitchell, 1995, Kargi and Curme, 1985, Lee *et al.*, 2011, Reu *et al.*, 1993 and Schulze, 1962). The use of a

rotating bioreactor for the culture of *Monascus* has not been reported before, and it would therefore be interesting to determine whether it is possible.

7.2 Results and discussion

7.2.1. Comparison of *Monascus* solid state culture on whole grain adlay and puffed adlay

In the first set of experiments (see 3.4.1.1 in Chapter 3), a comparison was made of the suitability of whole grain adlay or puffed adlay for the culture of *Monascus* culture in a rotating bioreactor. This was done by putting whole grain and puffed adlay in shake flasks both with a moisture content of 60%, and gently shaking the flask every day by hand for a few seconds. At the end of the experiments in both types of adlay angkak production, the pigment concentration, moisture content and stickiness were measured (see Figure 7.1 - 7.2).

It was found that the fermented kernels of the whole grain adlay could not be made to move relative to each other throughout the experiment because the grains had stuck together and to the glass. Fermentation therefore was slow, and progressed from the top of the substrate downwards. In fermented puffed adlay, however, the grains could be moved when the flask was gently shaken. Fermentation therefore started evenly across the bed. The exact reason for this difference in behaviour is unknown, but is most likely related to the porosity of the puffed adlay. A plausible mechanism is that in whole grain adlay the added water at first penetrated only the outer shell of the adlay grain, and only later, on during the cooking process, the inside layers. Gelatinization of the outer layer was therefore stronger, and the particle more sticky. In puffed adlay, grains have many cavities, and can absorb water in a few seconds (Mariotti *et al.*, 2006). The water could therefore penetrate directly into the whole of the particles, and gelatinisation during the cooking will be more even and lower at the surface than for the whole grains.

The conversion of adlay into pigment was much faster for puffed adlay than for whole grain adlay. This is clearly seen in Figure 7.1, where around seven days from the starting date no pigment can be seen in the flask with whole grain adlay, but pigment is being produced throughout the bed in the flask with puffed adlay.

Figure 7.1 Comparing *Monascus* culture on different types of adlay
(a) Fermented whole grain adlay (b) Fermented puffed adlay

When the pigment concentration was measured, however, it was found that puffed adlay had a slightly lower pigment concentration than the whole grain adlay (see Figure 7.2). This may be due to the fact that shear induced during the mixing process broke up the mycelia. Another plausible reason is that the moisture content was significantly reduced during the fermentation of the puffed grains, but less so of the whole grains. The reduced moisture content of the puffed grains may be due to the fact that the water evaporated more quickly from puffed grains, and the fact that a new fresh moist surface was exposed after each mixing step.

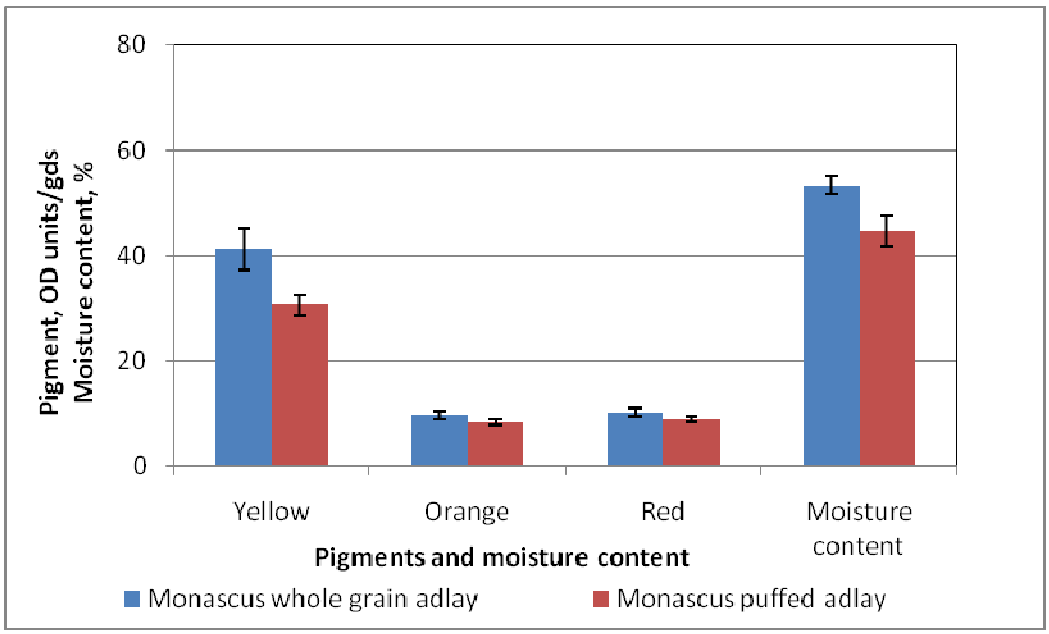


Figure 7.2 Comparison of the pigment concentrations and moisture content in fermented whole grain and puffed adlay

7.2.2. Effect of the addition of water to adlay

In the previous experiments, it was shown that the moisture levels had a significant effect on the ability of adlay grains to stick to each other, and also on pigment production. To investigate this further, different amounts of water were added to the whole grain adlay and the puffed adlay, and the mechanical properties of the materials were measured. In addition to this, the *Monascus* was cultured in flasks, without shaking, on puffed adlay with different water contents. After three weeks, the pigment and the glucosamine concentrations in the product were measured as well as the mechanical properties of the material.

Figure 7.3 shows the relation between the amounts of water added, pigment concentration and glucosamine concentration. A maximum is found in the concentration of the yellow pigment produced at 70% of water content whereas the maximium in the concentration of the orange and the red pigments could be found at 60% of water content. A maximum in the glucosamine concentration in the fermented adlay was found at a water content of 60%.

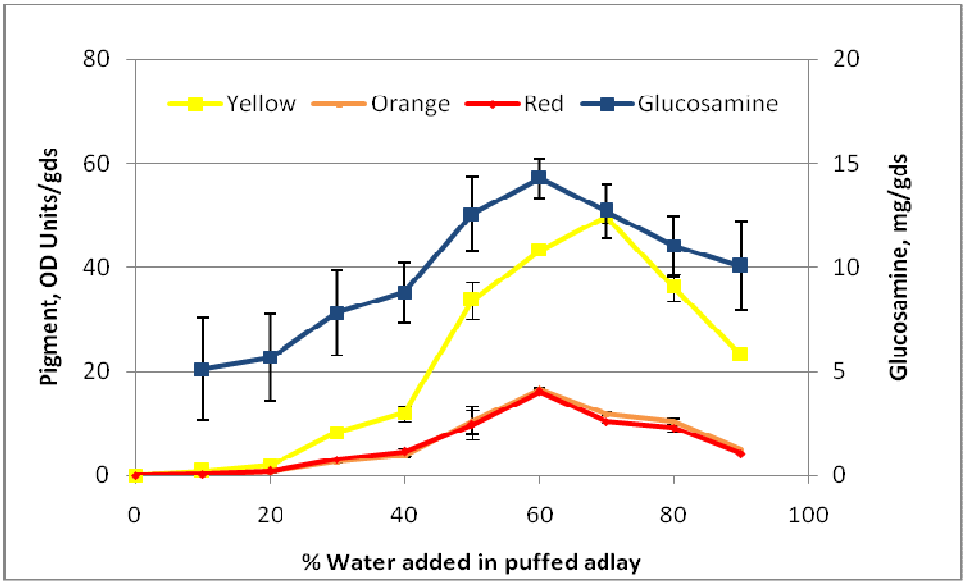


Figure 7.3 Pigment concentration and glucosamine concentration after fermentation of *Monascus* on puffed adlay with different amounts of water added

Remark: Three replicates are shown on the graph. The data of each replication averaged two times.

Figure 7.4, 7.5 and 7.6 shows the relation between the amount of water added and the mechanical properties of the product (hardness, cohesive strength and adhesiveness) for cooked whole grain adlay, cooked puffed adlay and fermented puffed adlay.

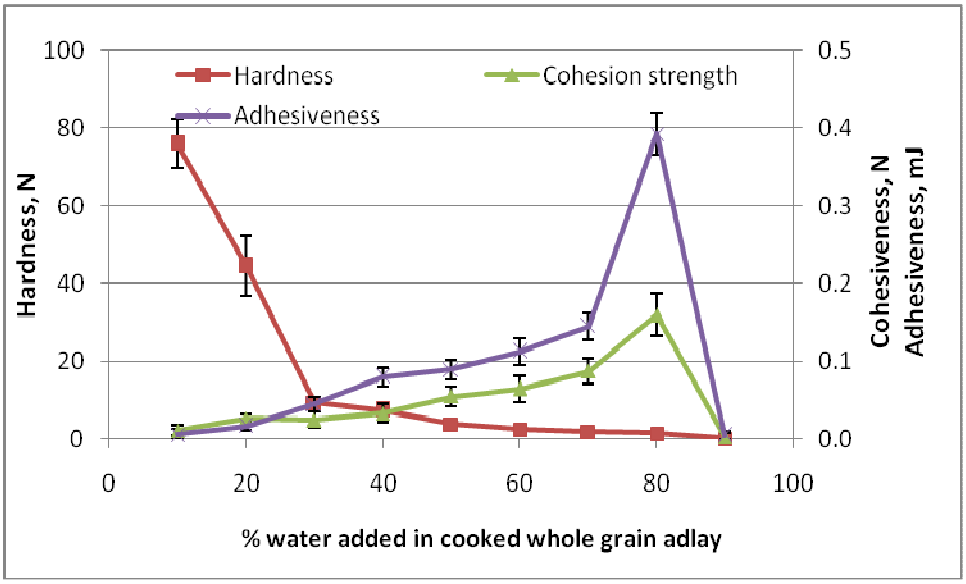


Figure 7.4 Mechanical properties of cooked whole grain adlay at different moisture contents

Remark: Data on the mechanical properties could not be obtained at a water content of 0% because the material's hardness was over 100 N. Three replicates are shown in the graph. Each replication was measured eight times on average.

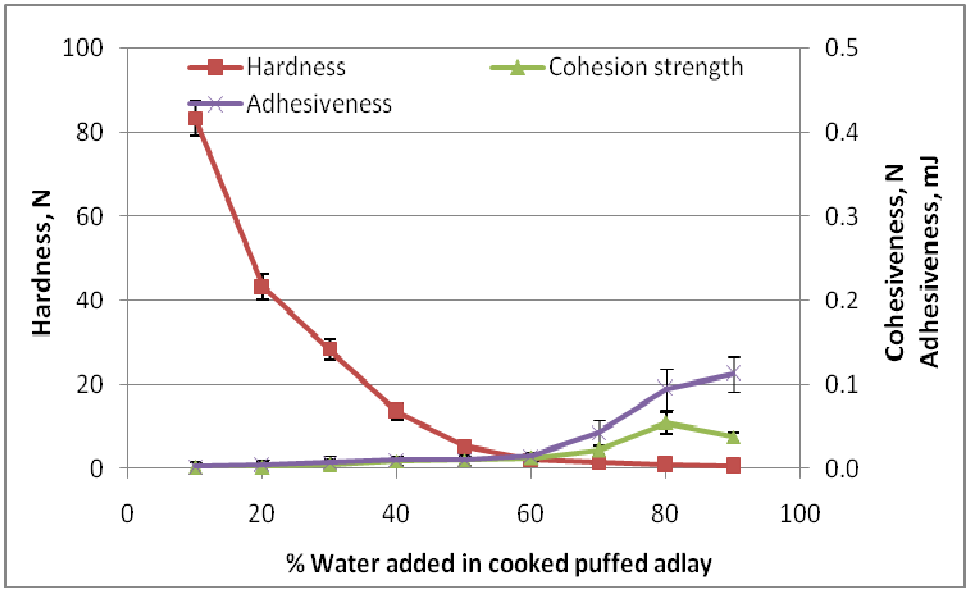


Figure 7.5 Mechanical properties of cooked puffed adlay at different moisture contents

Remark: Data on the mechanical properties could not be obtained at a water content of 0% because the material's hardness was over 100 N.

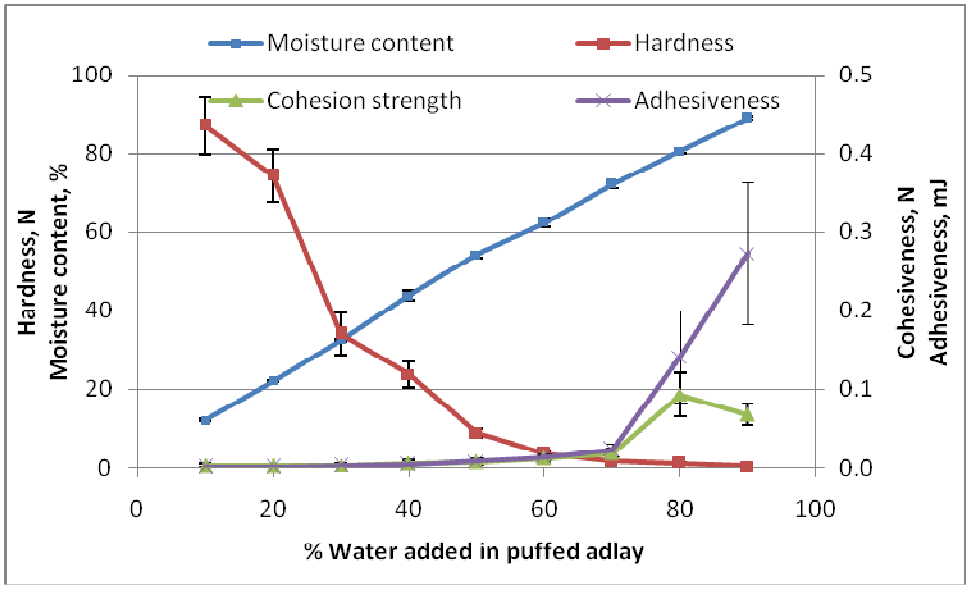


Figure 7.6 Mechanical properties and moisture content of adlay angkak made by growing *Monascus* on puffed adlay in a static chamber with different amounts of water

Remark: Data on the mechanical properties could not be obtained at a water content of 0% because the material's hardness was over 100 N.

The trends in the hardness of the three materials were similar, i.e. a steady decline in the materials'hardness with increasing moisture content. However, significant

differences can be seen in the cohesiveness of the materials, and even more in the adhesiveness.

In Figure 7.4 it can be seen that, at a water content of 60% to 80%, the cohesion strength and adhesiveness had increased to a much higher level than that of cooked puffed adlay and fermented puffed adlay. At a water content of 90%, the cohesion strength and adhesiveness of cooked whole grain adlay suddenly dropped to very low values. This was because at a water content of 90% the bed of whole grain adlay became flooded, making it much easier for the grains to move relative to each other. This did not happen with cooked puffed adlay and fermented puffed adlay. Cohesion strength and adhesiveness of fermented puffed adlay were higher than that of cooked unfermented puffed adlay, presumably because of the presence of mycelium.

Figure 7.6 also contains information about the moisture content. The total amount of water in the puffed adlay after fermentation was equal to the amount of water which was added, so little had been lost during the fermentation.

The largest difference in the mechanical properties of cooked whole grain adlay and unfermented and fermented puffed adlay can be seen at a moisture content of 60%, with puffed adlay having much lower cohesive strengths and adhesiveness. As this was also the moisture level at which the pigment production was highest, this moisture level was selected for the study for the culture of *Monascus* in a rotating bioreactor.

7.2.3. Effect of rotating speed on a culture of *Monascus* on puffed adlay in a continuously rotating bioreactor

Puffed adlay was placed in the chambers of rotating bioreactors with 60% moisture, autoclaved, and inoculated with *Monascus* (see 3.4.1.5 in Chapter 3). The chambers were then continuously rotated at rates from 0 to 16 rpm. After three weeks, pigment concentration, percentage of single particles, moisture content and mechanical properties of the fermented product were measured. The results are shown in Figures 7.7 and 7.8.

In Figure 7.7 it can be seen that the percentage of single grains or single particles (expressed as the ratio of the weight of the single particles to the total weight

of particles in the rotating chamber) was high at low rotating speeds but significantly decreased at medium and high rotating speeds. This decrease was due to the particles aggregating into a ball or balls when the rotating speed increased. A rotating speed of 0.25 rpm gave a percentage of single grains of around 80 ± 2.5 % whereas at 8 and 16 rpm it was 13.0 ± 0.5% and 6.0 ± 0.6% respectively. Clearly, at medium and high speeds the particles started to adhere to each other and formed aggregates. This increase in particles adhesion was arguably caused by the attrition of the particles by the continuous rotation. The moisture level was little affected. In a static chamber the moisture content was 65.5 ± 0.6%, but it decreased to 61.6 ± 1.1% at 0.25 rpm, and 62.4 ± 0.7 and 63.7 ± 0.7% at the high speeds of 8 and 16 rpm. The continuous rotation significantly affected the pigment concentration as well as the particles' mechanical properties. A static chamber gave 46.9 ± 2.7, 25.9± 3.7 and 25.0 ± 3.4 units per gram respectively, of yellow, orange and red pigments. At 0.25 rpm, the pigment concentration was just 32.3 ± 3.4, 8.8 ± 1.1 and 7.9 ± 0.8 units per gram respectively of yellow, orange and red pigments. When the rotating speed had increased, the particles aggregated together. Although this aggregation reduced the oxygen transfer, it also reduced the effects of the shear rate on the mycelium and allowed more water to remain trapped. The pigment values increased slightly to reach 35.0 ± 2.1, 11.5 ± 1.9 and 10.4 ± 1.7 units per gram respectively of yellow, orange and red pigments at a medium speed of 8 rpm and 43.4 ± 5.7, 14.3 ± 3.7 and 13.0 ± 3.9 units per gram respectively, with yellow, orange and red pigments at a high speed of 16 rpm. The findings are similar to other observations with rotating bioreactors. For example, Mitchell *et al.* (2000) reported that particles in a rotating bed bioreactor can knit together when the bed is rotated continuously at high speeds. Stuart *et al.* (1999) monitored the oxygen uptake rate during a fermentation of *Aspergillus oryzae* on wheat bran in rotating drum bioreactor and found that shear effects at rotational speeds over 30 rpm reduced productivity or sporulation.

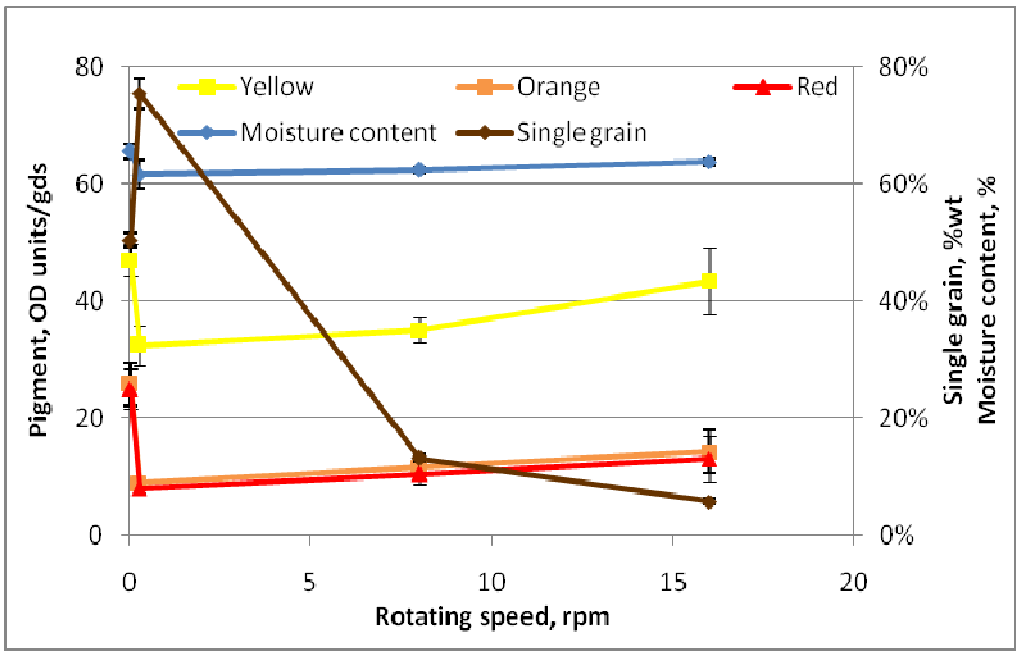


Figure 7.7 Pigment values, moisture content and percentage of single grains after the culture of *Monascus* on puffed adlay in a continuously rotating bioreactor

In Figure 7.8, the mechanical properties of fermented products are shown. The hardness of the particles appears to decrease with increasing speed of rotation of the chamber (though not significantly) but the cohesion strength and adhesiveness of the particles increased significantly as the rotating speed increased.

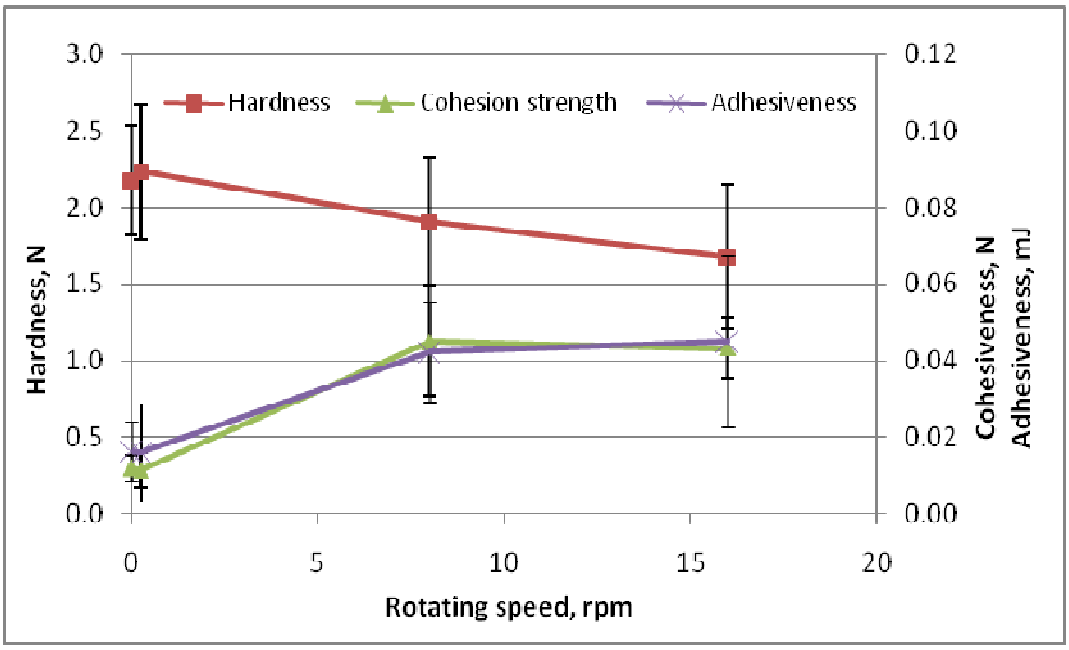


Figure 7.8 Changes in the mechanical properties of *Monascus* puffed adlay during a continuously rotating culture

The findings that when high rotation rates are used the particles stick together are not new. For example, Pandey (1991) reported that it is commonly found that when rotating beds are used for fungal fermentation a high rate of rotation causes particles in the bed to aggregate into balls. Pandey also reported that a gentle continuous rotation was often better because high rates damaged mycelia. The experiment was found instead that ball formation gave higher rates of production in continuously rotated vessels.

7.2.4. Effect of rotating speed and frequency of rotation on a culture of *Monascus* on puffed adlay in an intermittently rotating bioreactor

In the next set of experiments, *Monascus* was again cultured on puffed adlay in a rotating bioreactor (see 3.4.1.5 in Chapter 3), but this time the chamber was only rotated for a short time (3 minute) at regular intervals during the day. The reason for trying this regime was to find out whether the short period of rotation would be enough to mix the fungus over the chamber and give a homogeneous product, but short enough not to cause extensive damage to the surface of the particles (leading to aggregation) or to the mycelia (leading to reduced production). The range of rotating speeds to be tried was between 0 to 16 rpm and the frequency of operation between 0 and 8 times per day. After three weeks, the pigment concentration, moisture content and percentage of single particles in the fermented product were determined as well as the mechanical properties of the product. The results are shown in Figures 7.9 to 7.14.

The results obtained with a frequency of operation of 8 times per day are shown in Figure 7.9 for different rates of rotating speed. Rotating a chamber at low speed several times a day appears to increase the number of single grains in a bed, but doing it too fast appears to negate this effect again. The pigment production decreased significantly compared with a static culture at low rotation rates but increased again when higher rotation rates were used. Figure 7.10 shows the mechanical properties of the fermented material. There is little change in the mechanical properties of the bed material.

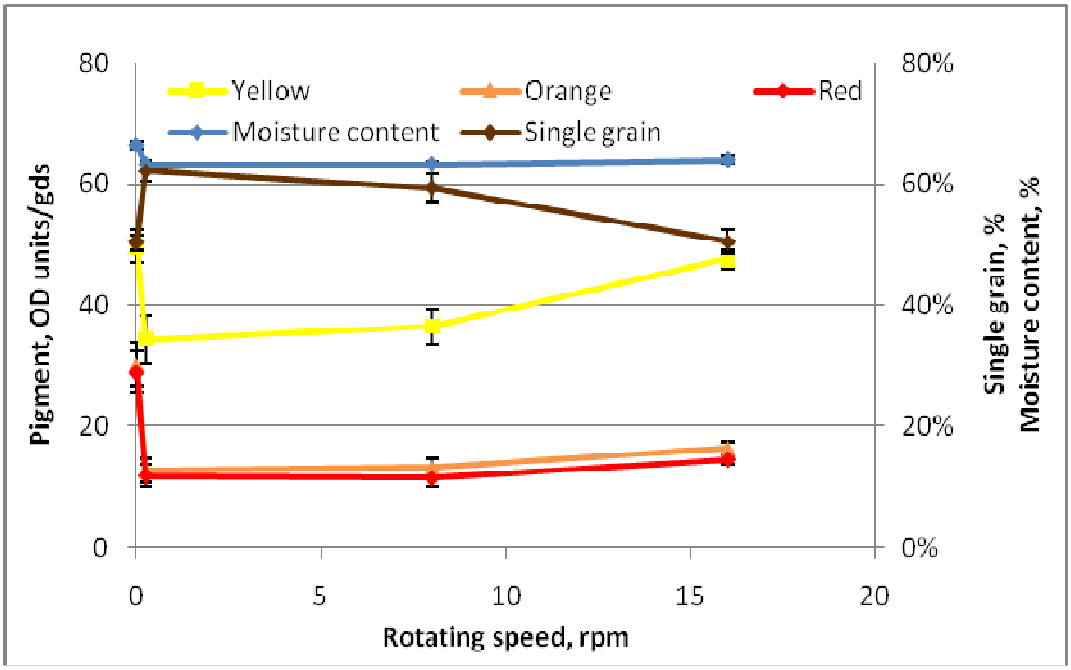


Figure 7.9 Pigment concentration, moisture content and percentage of single grains after the culture of *Monascus* on puffed adlay in an intermittently rotating bioreactor. The frequency of the periods of rotation (3 min) was 8 times per day, and the rotation rates between 0 and 16 rpm.

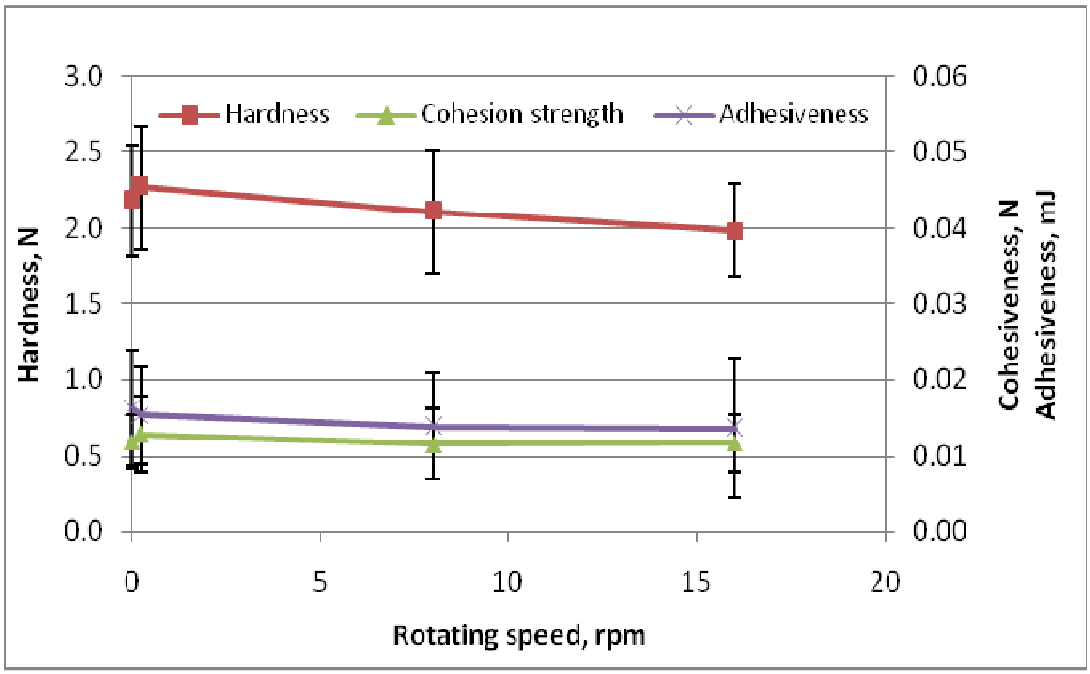


Figure 7.10 Mechanical properties of the bed material after the culture of *Monascus* on puffed adlay in an intermittently rotating bioreactor. The frequency of the periods of rotation (3 min) was 8 times per day, and the rotation rates between 0 and 16 rpm.

Results obtained at a medium frequency of rotation at 3 times per day are shown in Figure 7.11 and 7.12. The trend in the number of single grains in the bed is similar to that found when a high frequency of rotation of 8 times a day was used. The change in moisture content is minimal gain. However, this time the pigment concentrations in the final product are very similar even when the chamber is rotated at different rotation speeds. In Figure 7.12, the mechanical properties of the bed material are shown. The mechanical properties of the bed material appear to change little.

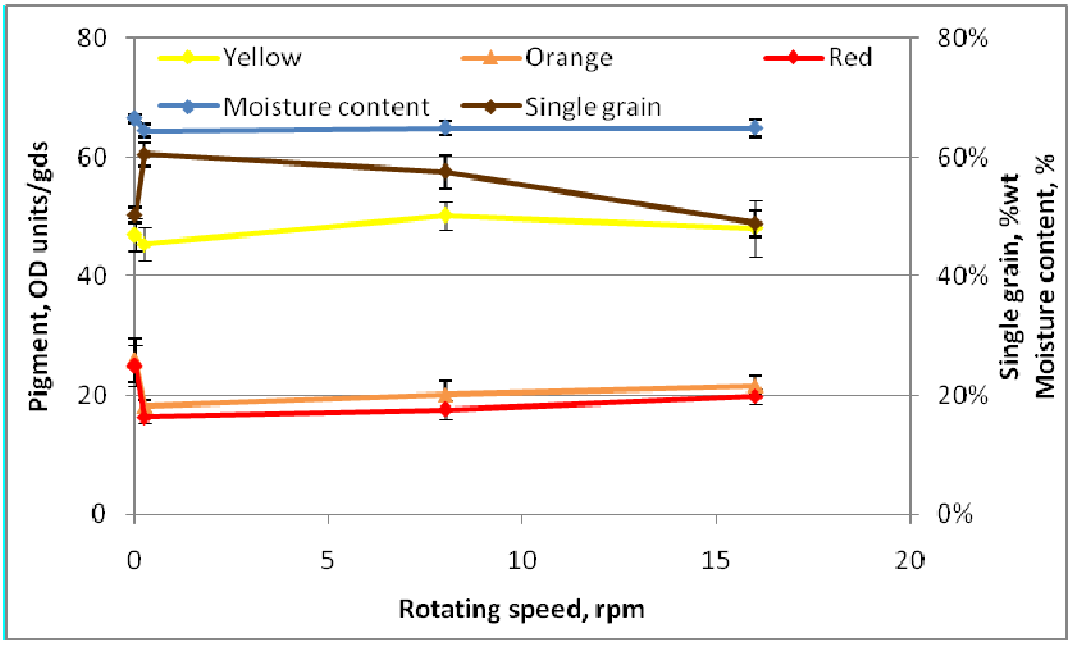


Figure 7.11 Pigment concentration, moisture content and percentage of single grains after the culture of *Monascus* on puffed adlay in an intermittently rotating bioreactor. The frequency of the periods of rotation (3 min) was 3 times per day, and the rotation rates between 0 and 16 rpm.

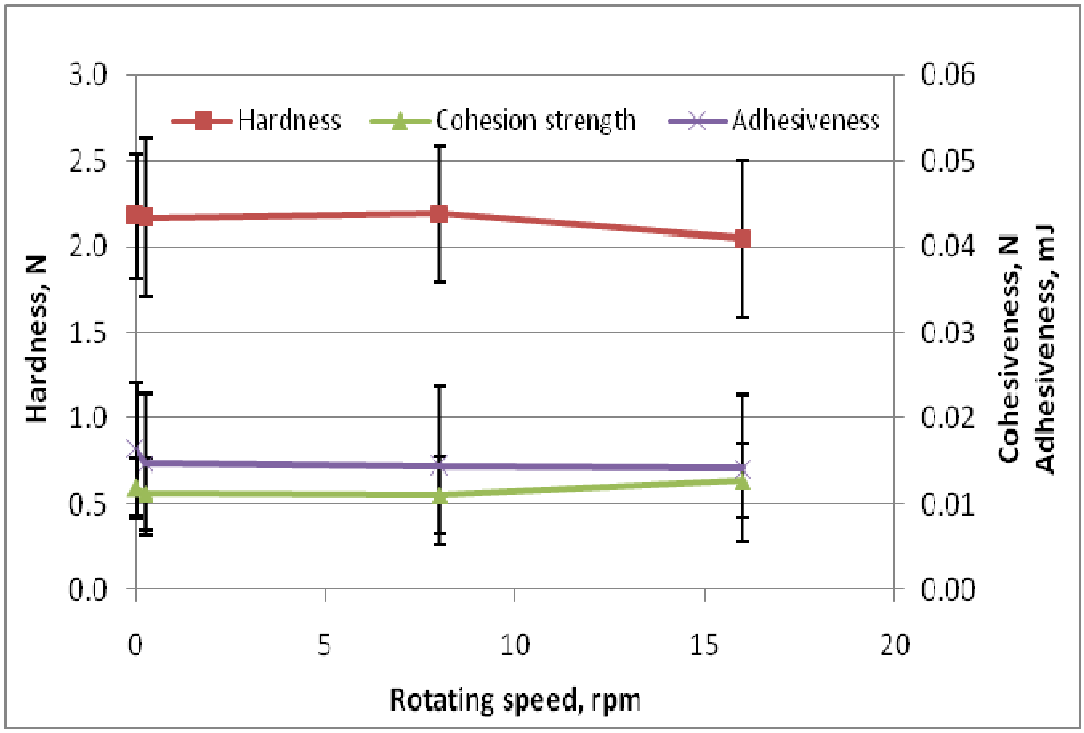


Figure 7.12 Mechanical properties of the bed material after the culture of *Monascus* on puffed adlay in an intermittently rotating bioreactor. The frequency of the periods of rotation (3 min) was 3 times per day, and the rotation rates between 0 and 16 rpm.

In the final experiment, a low frequency at 1 period of rotation per day was used. The results are shown in figure 7.13 and 7.14. In Figure 7.13, it can be seen that the percentage of single grains slightly increased compared to a static culture when a scheme of rotation was implemented but decreased slightly when higher speeds of rotation were used. Moisture content remained constant. Final pigment concentrations also changed little, with only a slight increase in the production of yellow pigment from 45.4 ± 0.7 units per gram at low speeds to 50.5 ± 1.9 units per gram at the highest speed. In Figure 7.14, the mechanical properties of the fermented product are shown. Little change can be seen in the mechanical properties of the bed material.

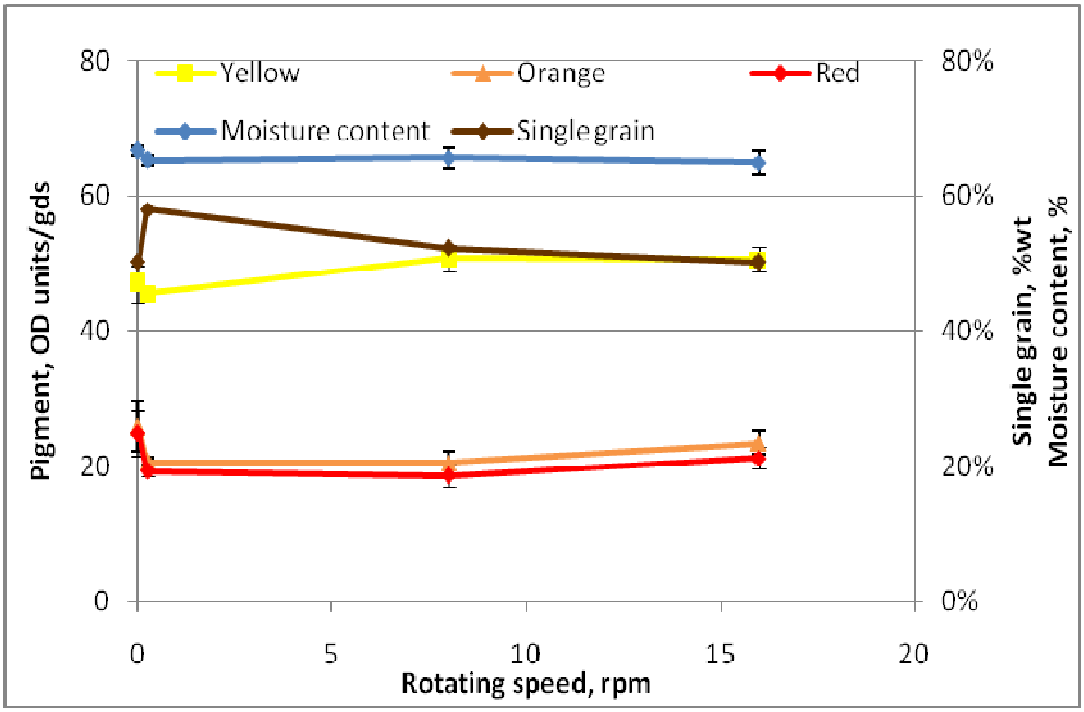


Figure 7.13 Pigment concentration, moisture content and percentage of single grains after the culture of *Monascus* on puffed adlay in an intermittently rotating bioreactor. The frequency of the periods of rotation (3 min) was once per day, and the rotation rates between 0 and 16 rpm.

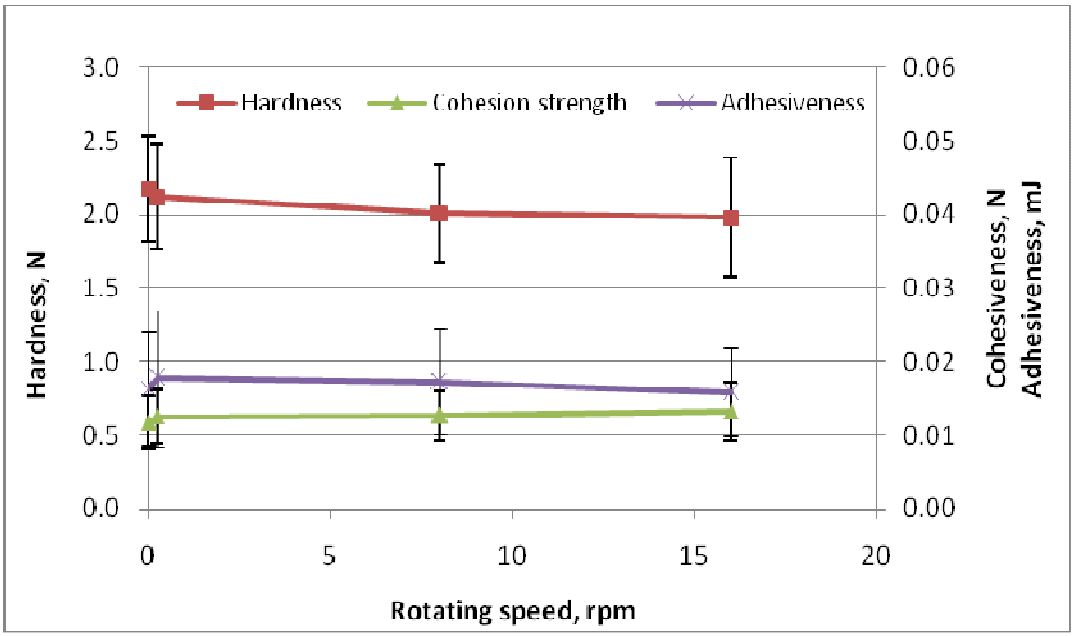


Figure 7.14 Mechanical properties of the bed material after the culture of *Monascus* on puffed adlay in an intermittently rotating bioreactor. The frequency of the periods of rotation (3 min) was once per day, and the rotation rates between 0 and 16 rpm.

The results obtained indicate an intermittently rotating drum bioreactor is better than a continuously rotating bioreactor. Whilst rotation is beneficial, it should be done sporadically and a low speed of rotation should be used. These findings are similar to those observed in other fungal fermentations in drum bioreactors. For example Lee *et al.* (2011) reported that when a fungal fermentation was done on palm kernel cake in a rotating drum bioreactor, the highest cellulose activity was obtained with a low speed at 0.5 rpm and mixing intensity of once every 24 hours.

7.3. Conclusions

The fact that cooked puffed adlay was much less sticky than whole grain adlay, and could be readily mixed is of significant potential benefit for the production of angkak by *Monascus*. It allows the inoculum to be evenly spread over the substrate bed, making it possible to have the fermentation process start evenly across the bed. The mixing also reduces transport problems in the bed, giving a more even distribution of heat, moisture, nutrients and oxygen in the bed and giving a more homogeneous product. A rotating drum bioreactor has been found to be suitable for the mixing and the culture. Continuous rotation caused damage to mycelia. At high rotation rates it also caused grains to stick to each other, resulting in the formation of balls of aggregated grains in the reactor. Although this ball formation had some benefits in the small reactor used, at larger scales this would most likely lead to issues. Problems with ball formation were not seen in rotating drum bioreactors in which the reactor was only rotated for a brief period once or a few times a day. An intermittently rotating drum bioreactor appears to be the better option. It gave a lower percentage of single particles but a higher pigment yield.

CHAPTER 8

ENHANCED PRODUCT FORMATION DURING SOLID STATE CULTURE OF *MONASCUS* BY ACOUSTIC VIBRATION

8.1 Introduction

Angkak is a natural red colourant which is usually made by culturing a *Monascus spp.* on cooked rice, though other materials are also used (Babitha *et al.*, 2007, Carvalho *et al*., 2007, Lee *et al.*, 2006, Lee *et al.*, 2008, Lin and Lizuka, 1982, and Velmurugan *et al.*, 2011). *Monascus* can produce coloured pigments which are yellow, orange, and red colours (Rosenblitt *et al.*, 2000). Angkak has been used as a natural pigment in fish (surimi and other marine products), Chinese cheese, red wine, tomato ketchup and meat products (sausages and hams).

Pigment production can be divided into two processes: solid state and submerged culture. Higher productivity can be achieved with a solid state fermentation of *Monascus* without mechanical aeration of pigments than with submerged culture (Carvalho *et al*., 2006 and Yang *et al.*, 2005). Moore *et al.* (2001) and Wang *et al.* (2007) reported that solid state culture gave more than ten times more pigment than submerged culture. Solid state culture takes longer, due to the low availability of free water in the substrate material (Singhania *et al.*, 2009). The reaction rate in the solid state fermentation is often controlled by oxygen diffusion and oxygen consumption (Doran, 2013, Rahardjo *et al.*, 2006 and Viccini *et al.*, 2001). Two different zones can be distinguished. The first is oxygen transfer into the bed of material. This can be increased by ensuring the exchange of and continuous renewal of air at the surface of the material and the surface of the bed. The other is oxygen transfer into the particles. This can be increased by enlarging increasing particle porosity or reducing the particle size (Pandey *et al.*, 2000, Raghavarao *et al.*, 2003 and Viccini *et al.*, 2001).

In chapter 7, it was shown that the use of puffed adlay, which has a higher porosity than whole grain, could increase pigment production in a fixed tray fermenter by 10 times. In chapter 4, however, when it was attempted to increase the surface area

by using ground grain, it was shown that production was actually reduced and remained limited to a small area near the top surface. The reason for this was thought to be the fact that the particles of ground adlay were quite small and sticky, and that little air space was present in the bed, causing there to be transport limitation in the bed, in particular of oxygen. In this chapter, it will be explored whether transport within the bed material can be increased by mechanical means, and in particular by using acoustic vibration (sound). The use of acoustic vibration over other mechanical means is that it involves less moving parts (no motor, rotors, seals, and grease are needed), and the lifetime of equipment is longer.

Sound consists of oscillating pressure waves which are transmitted through solid, liquids or gases. The sound waves, when travelling through a medium, suffer changes in their properties (e.g. velocity, attenuation, frequency, spectrum,.....) which can be used for measurements; the sound energy also can induce movement of material (e.g. acoustic streaming) or induce chemical reactions (sonochemistry) (Awad *et al.*, 2012, Mason and Lorimer, 2002, Lorimer and Mason 1987, Patist and Bates, 2008, Patist and Bates, 2011, Soria and Villamiel, 2010 and Sulaiman *et al.*, 2012). For audible sounds the vibration of molecules around their balance position is sufficiently strong to stimulate mechano-sensory cells for the vibrations to be detected by the human ear, i.e. they can be heard. The typical range of audible sound is between 20 Hz and 20 kHz. Lower frequencies (large wavelengths) are referred to as infrasound and higher frequencies (smaller wavelengths) as ultrasound (Aggio *et al.*, 2011 and Cárcel *et al.*, 2012).

Ultrasound waves occur at a frequency above the threshold of human hearing. They can be divided into three frequency ranges: power ultrasound or conventional power ultrasound (high power ultrasound, 20 - 100 kHz), high frequency ultrasound (medium power ultrasound, 100 kHz - 1 MHz) and diagnostic ultrasound (low power ultrasound, >1MHz). Medical imaging operates at frequencies in the higher megahertz range and the intensity of power applied is typically less than 1 W/cm^2 (Cárcel *et al.*, 2012, Lorimer and Mason, 1987, Mason, 1998 and Muthukumaran *et al.*, 2007). Sonochemistry is typically done in the frequency range from 20 kHz to ca 2 MHz.

Low frequency high power ultrasound typically involves the use of powers in the range 10 - 1000 W/cm^2). Under these conditions large cavitation bubbles are

produced. As the frequency increases cavitation becomes less violent. In the megahertz range there is no cavitation and the main mechanism movement is acoustic streaming.

Cavitation is the formation, growth, and implosive collapse of air bubbles and may occur when small air bubbles (vapour bubbles, or holes) form in the liquid phase. Cavitational collapse produces intense local heating (~5000 K), high pressure (~1000 atm), and enormous heating and cooling rates (>109 K/sec) and liquid jet streams (~400 km/h). The intensity of cavitation and its effects depend on the characteristics of the medium, such as viscosity, and/or process variables, like ultrasonic intensity, ultrasonic frequency or pressure. Higher amplitudes result in a more effective creation of cavitation. For example, a "Hielscher" device can produce amplitudes of up to 115 □m. These high amplitudes allow for a high power transmission ratio that in turn allows the creation of high power densities of up to 100 W/cm^3 (Cárcel *et al.*, 2012, Chisti, 2003, Hielscher, 2005, Knorr *et al.*, 2004, Mason *et al.*, 1996, Mason, 1998, Rokhina *et al.*, 2009, Suslick, 1998 and Yang *et al.*, 2005). Ultrasonically induced high intensity cavitation is a proven technology for cleaning mechanical parts or electronic circuit boards. It also has potential applications in food surface washing (e.g. decontamination of fresh produce), extraction of antioxidants from plant tissues, cell disruption, dehydration, germination of plant seed, liquid degassing, homogenization of emulsion, pasteurization and sterilisation. Ultrasound can remove lipids more effectively than mechanical and manual shaking (Awad *et al*., 2012, Chemat *et al.*, 2011, Knorr *et al.*, 2003, Mason *et al.*, 1996, Matsuura *et al.*, 1994, Schläfer *et al.*, 2002 and Wambura *et al*., 2010).

Ultrasound can have strong biological effects. Cavitation may denature proteins, lead to the damage of the cell wall and cause irreversible changes in permeability of the cell membrane. Ultrasound may also impose a hydrostatic pressure on the cell membrane which breaks the cell membrane. The effects of low intensity ultrasound can, however, be reversible. Low intensity ultrasound can improve the mass transfer of reagents and products through the boundary layer or through the cellular wall and membrane (Kwiatkowska *et al*., 2011, Pitt and Rodd, 2003, Sala *et al.*, 1995, Sinisterra, 1992, Swamy *et al.*, 2005, Villamiel and de Jong, 2000 and Yang *et al.*, 2005). Thus, there is much evidence for beneficial effects of controlled sonication of live cells. For example, Swamy *et al.* 2005 reported that heterotrophic growth of bacteria and fungi on solid substrates could be improved by controlled exposure to a low dose of ultrasound.

Aggio *et al*. (2011) compared the physiology of yeast cells growing in a defined liquid medium exposed to music, high and the low frequency sonic vibration, and silence. Sonic stimulations not only increased the growth rate of the yeast cells by 12% but they also reduced biomass production by 14%. Different metabolic pathways were affected by different sound frequencies. Matsuhashi *et al.* (1998) also reported positive effects of sound waves on bacterial cells. Yang *et al.* (2005) reported that *Monascus* pigment production on glucose medium could be increased by 30% with a low dose of low frequency ultrasound with a power of 200 Watts ultrasound for a short period of time (2 min) every six hours. The application of ultrasound to solid state fermentation of *Monascus*, however, to our knowledge has not been reported before.

In this chapter, it is discussed whether pigment production by *Monascus* during solid state fermentation can be enhanced by using acoustic vibration. Both audible waves and high frequency ultrasound is explored.

8.2. Results and discussion

8.2.1 A comparison of the culture of *Monascus* on whole adlay and ground adlay

In the first set of experiments, a comparison was made between whole grain and the ground grain in the 250 mL Erlenmeyer flask (see 3.4.1.1 in Chapter 3). In Figure 8.1 and 8.2, a comparison is made between the pigment concentration and the moisture content of the untreated cooked whole grain of adlay, cooked ground grains of adlay, and their fermented products. The culture period was three weeks.

As expected untreated cooked whole grain adlay and ground adlay contained little or no pigment, and the fermented product had significant amounts. The fermentation on ground adlay produced significantly less pigment than the culture on whole grain adlay. The moisture content changed little.

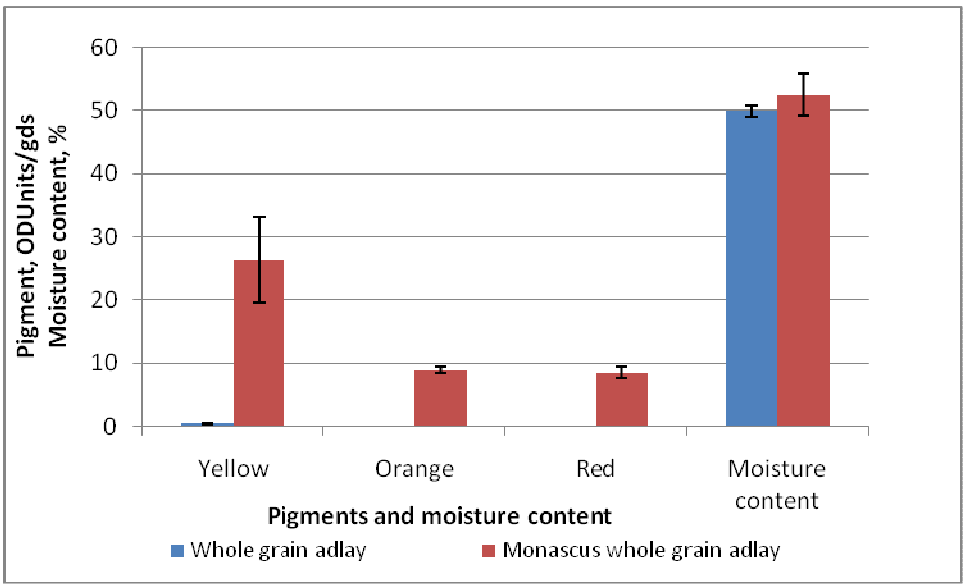


Figure 8.1 Pigment products and moisture content on whole grain adlay and its products

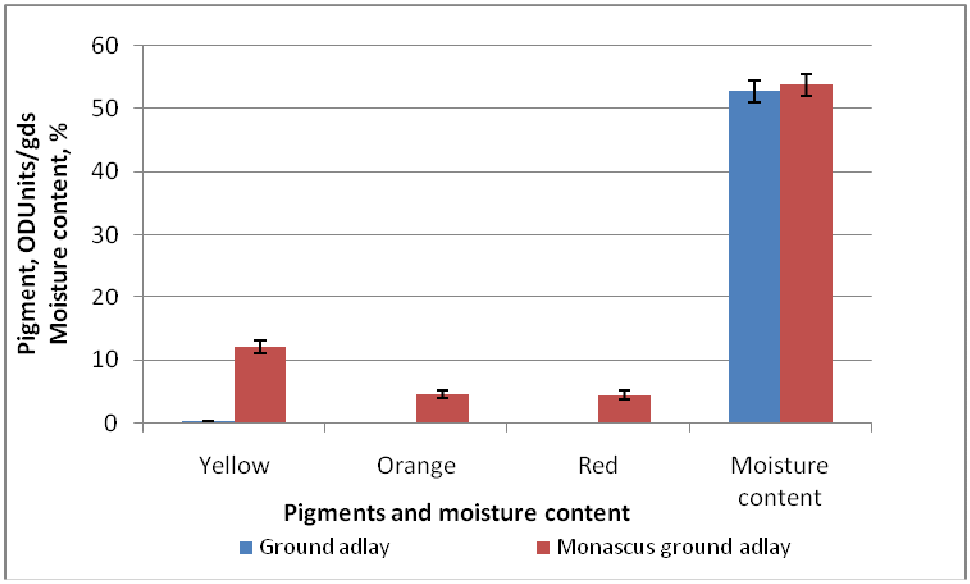


Figure 8.2 Pigment product and moisture content on ground adlay and its products

In Figures 8.3 and 8.4, a comparison is made between the mechanical properties of cooked whole grain and ground adlay and their fermented products. Cooked whole grain adlay had values of hardness, cohesion strength and adhesiveness at 3.80 ± 0.77 N, 0.06 ± 0.02 N and 0.11 ± 0.04 mJ before fermentation; after fermentation they were 3.09 ± 0.37 N, 0.01 ± 0.01 N and 0.02 ± 0.01 mJ, respectively. The cooked ground adlay had values of 6.78 ± 2.17 N, 0.61 ± 0.32 N and 0.86 ± 0.56 mJ respectively, for hardness, cohesion strength and adhesiveness. In the fermented product by close to 50% moisture content, these values had decreased to 3.43 ± 0.78 N, 0.28 ± 0.14 N and 0.32 ±

0.25 mJ, respectively. Comparison of the materials shows that cooked ground adlay is stickier, presumably because of its large surface area and gelatinisation of the starch is quicker. The hardness and stickiness in both materials had decreased after fermentation, but the ground adlay had decreased more significantly. The measurement of the mechanical properties of cooked whole grain and ground adlay therefore confirmed previous observations, and also that mixing during solid state culture with standard methods (e.g. rotating drum culture) of *Monascus* on ground adlay would be difficult as the particles would just congeal into a large ball.

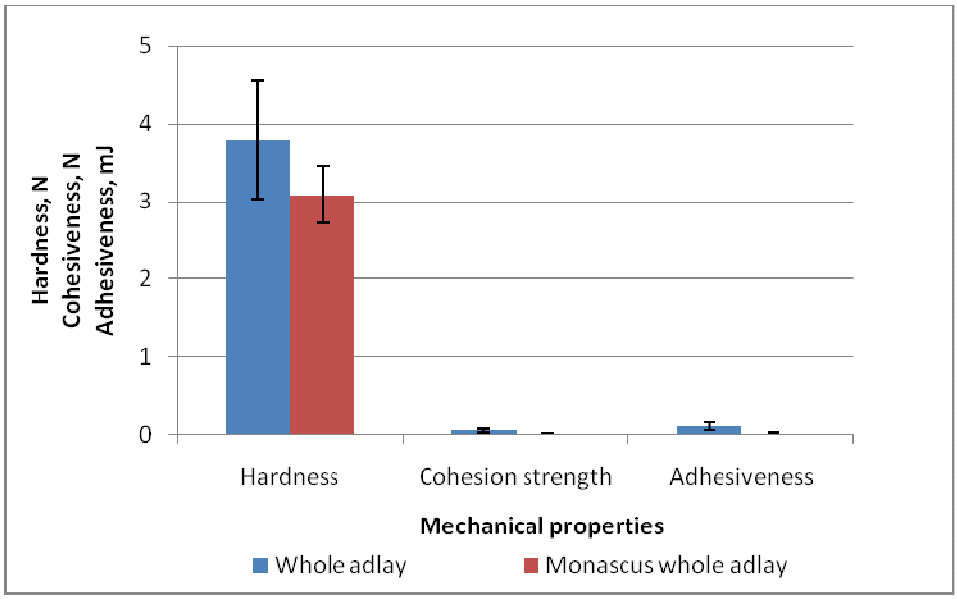


Figure 8.3 Mechanical properties of cooked whole grain adlay and its fermented product

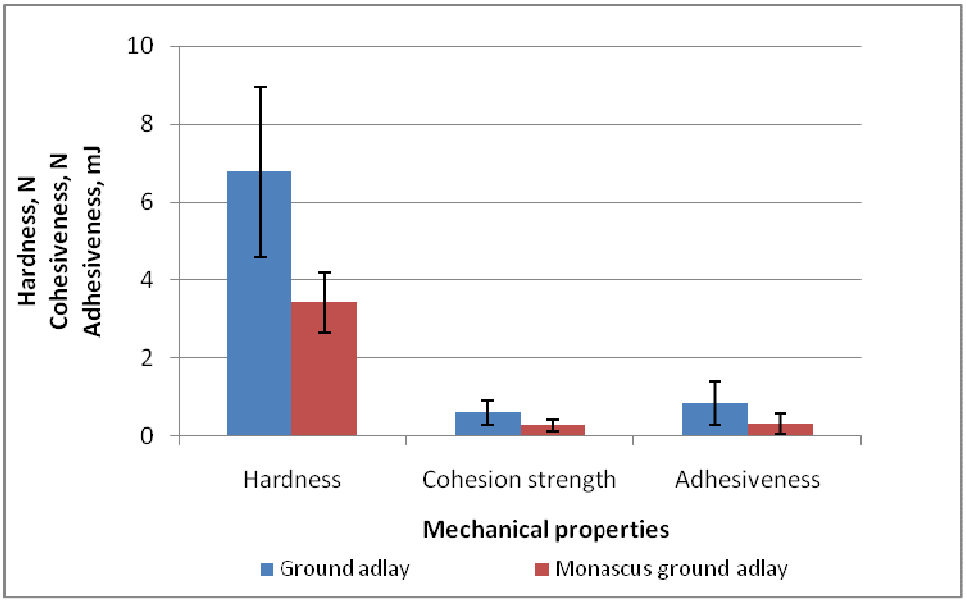


Figure 8.4 Mechanical properties of cooked ground adlay and its fermented product

8.2.2. Effect of sound frequency in *Monascus* ground adlay culture

Audible sound was applied to a thin layer of ground adlay (~ 1 cm) for 15 minutes each day for three weeks. A frequency range of 40 to 160 Hz frequency was selected as this gave a strong vibration of the microphone used to produce the sound. A frequency at 40 Hz gave the strongest vibration. The vibration declined when increasing the frequency from 40 to 160 Hz (see 3.6.1 in Chapter 3).

Figure 8.5 shows the pigment production and the moisture content after the exposure of the cultures to audible sound of different frequencies. The moisture content changed little. A frequency at 40 Hz increased pigment production relative to that of the controlled sample. When the frequency was increased from 40 to 160 Hz, pigment production slightly decreased; this may be due to the fact that the strength of the vibration decreased.

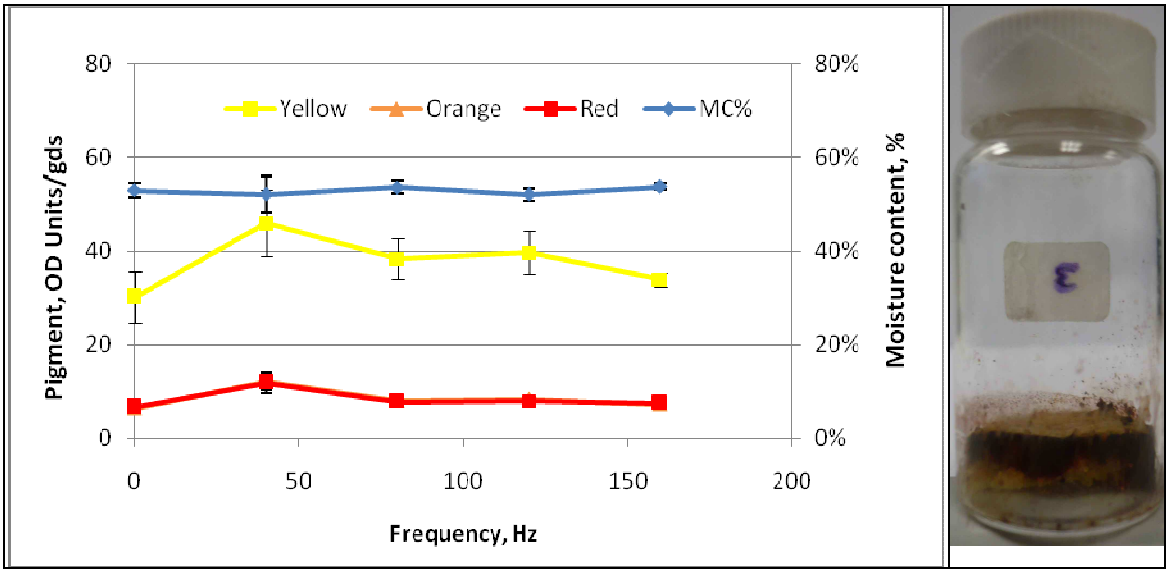


Figure 8.5 Effect of frequency of sound on pigment production by *Monascus* during a culture on ground adlay. The exposure to ultrasound was daily for 15 min for three weeks.

The glucosamine content of the product can be used as a measure of the biomass concentration. Figure 8.6 shows the glucosamine content is hardly affected by the application of sound at different frequencies, with a small (but not significant) increase at 40 Hz.

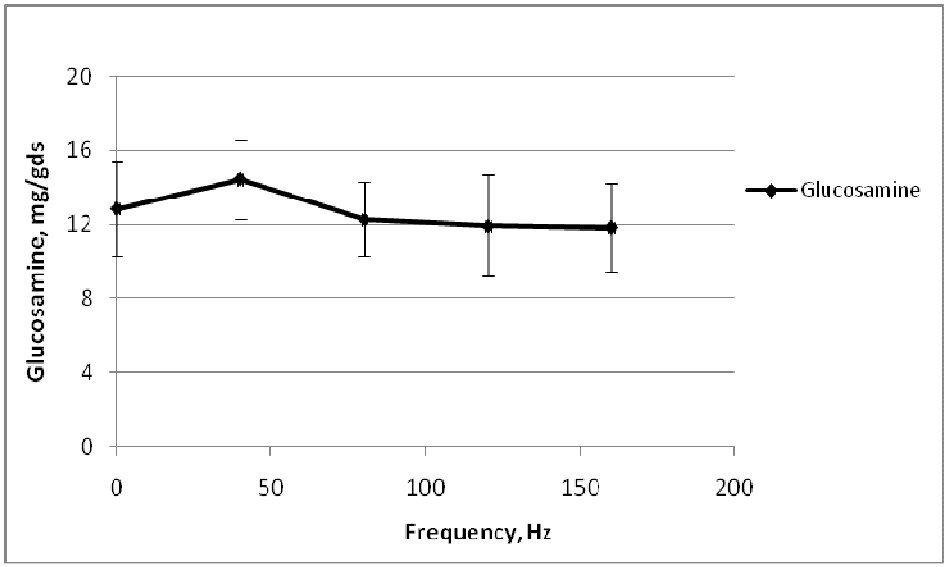


Figure 8.6 Glucosamine concentration in angkak made from ground adlay after daily treatment with audible sound for 15 minutes for three weeks

When the material was harvested, an area was found in the middle of the material that still had no colour. The vibration increased pigment production only on the surface. Overall, the benefit of exposing the sample to audible sound was unclear. The intensity of the vibration did not appear enough to move the quite sticky particles relative to each other. Increasing the intensity might well have caused stress for the cells and interfered with cell growth and pigment production.

8.2.3. Effect of the ultrasound power on the culture *of Monascus* on ground adlay

In this experiment, ultrasound of a high frequency (1.080 MHz) was applied to a thin layer of the ground adlay (~ 1 cm) on which *Monascus* was grown, for a short period of time every day for three weeks. It was expected that at this high ultrasonic frequency cavitation would be lower. The power supplied to the piezo-ceramic was varied between 0 and 12 Watts (see 3.6.2 in Chapter 3).

Figure 8.7 shows pigment development and moisture content after three weeks of the culture of *Monascus* on ground adlay after daily exposure of the adlay to ultrasound for 15 minutes at different powers. The moisture levels were maintained at low power but declined when powers over 6 Watts were used. This clearly indicates that

significant heating was produced during the exposure of the samples. The pigment concentration increased compared to the control when the power was increased from 0 to 3 Watts but decreased again when the power was more than 6 Watts. This decline was most likely related to the loss of moisture. Pigment distribution in the sample obtained at 3 Watts was homogeneous.

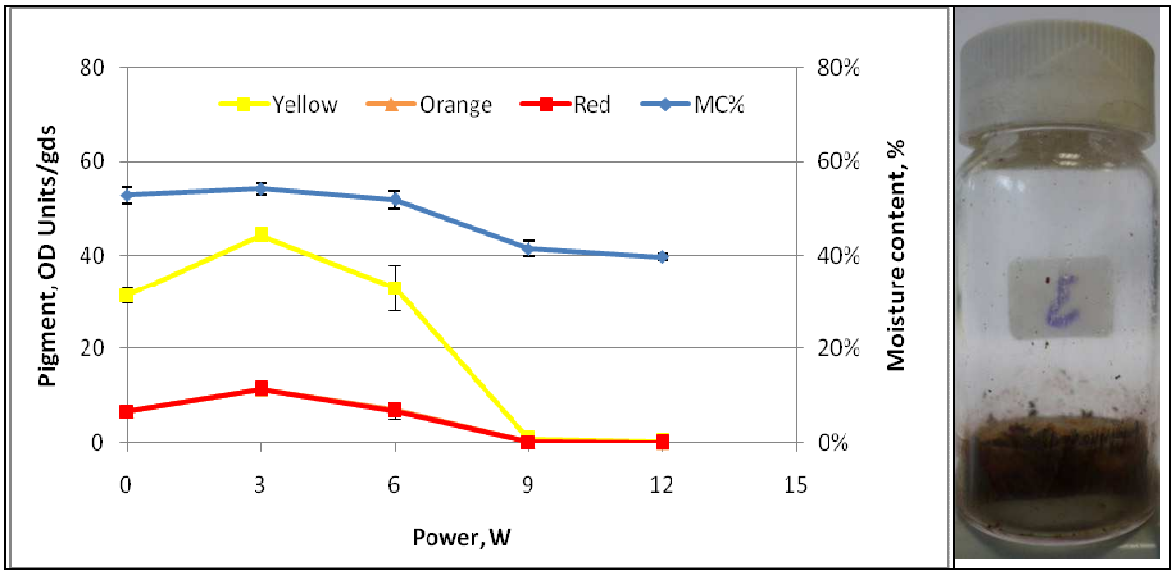


Figure 8.7 Study of the effect of ultrasound power on a culture of *Monascus* on ground adlay. The ultrasound had a frequency of 1.080 MHz, and the exposure time was 15 minutes per day for three weeks.

Figure 8.8 shows the change in the glucosamine content of adlay angkak of the same samples. The change in the biomass follows the same pattern as the pigment production.

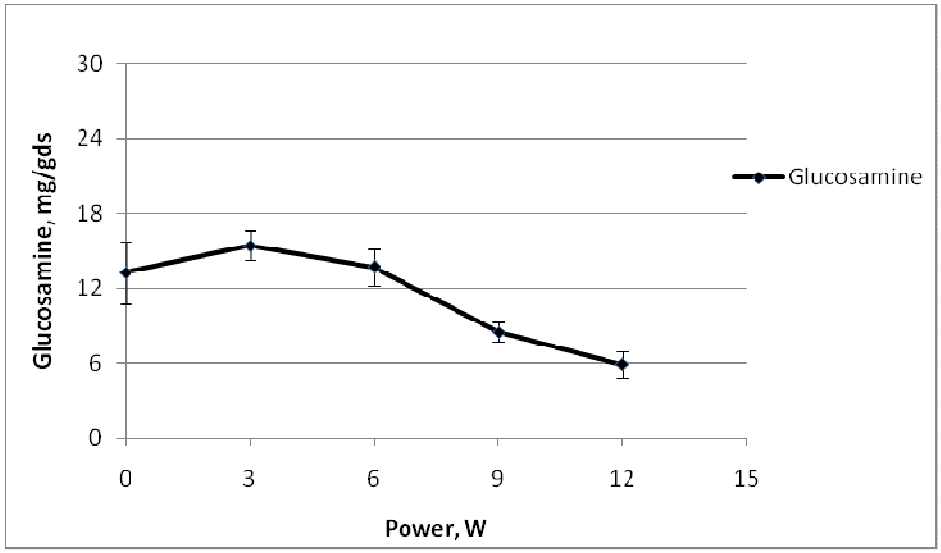


Figure 8.8 Change in the glucosamine concentration of angkak made from ground adlay after the exposure of the culture to ultrasound of 1.080 MHz and different powers for 15 minutes per day for three weeks.

Figure 8.9 shows the pigment development and the moisture content when applying ultrasound daily for 10 minutes. The moisture content still declined when the power was increased to over 9 Watts. The pigment production significantly increased with power to reach a maximum of 6 Watts. The subsequent decline at the higher powers of the ultrasound is most likely related to the drop in moisture level. Figure 8.10 shows the glucosamine concentration in the samples after exposure to ultrasound of different powers daily for 10 minutes. Biomass levels appear to reach a peak around 6 Watts, which coincides with the peak in pigment production. When the fermented product was harvested, the pigment was found spread evenly over the bed material in the sample found at 6 Watts. Only a small amount of the pigment could be found at higher power levels.

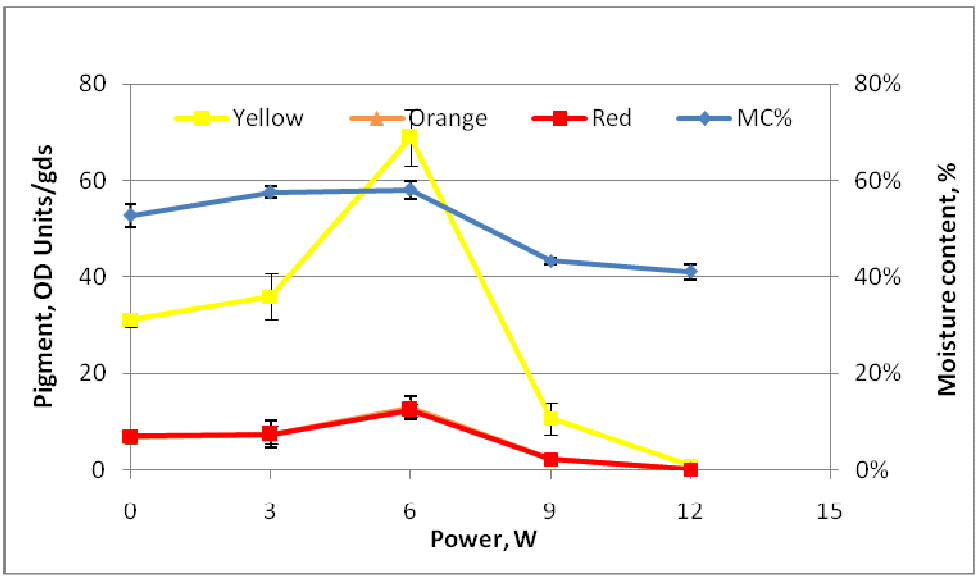


Figure 8.9 Study of the effect of ultrasound power on a culture of *Monascus* on ground adlay. The ultrasound had a frequency of 1.080 MHz, and the exposure time was 10 minutes per day for three weeks.

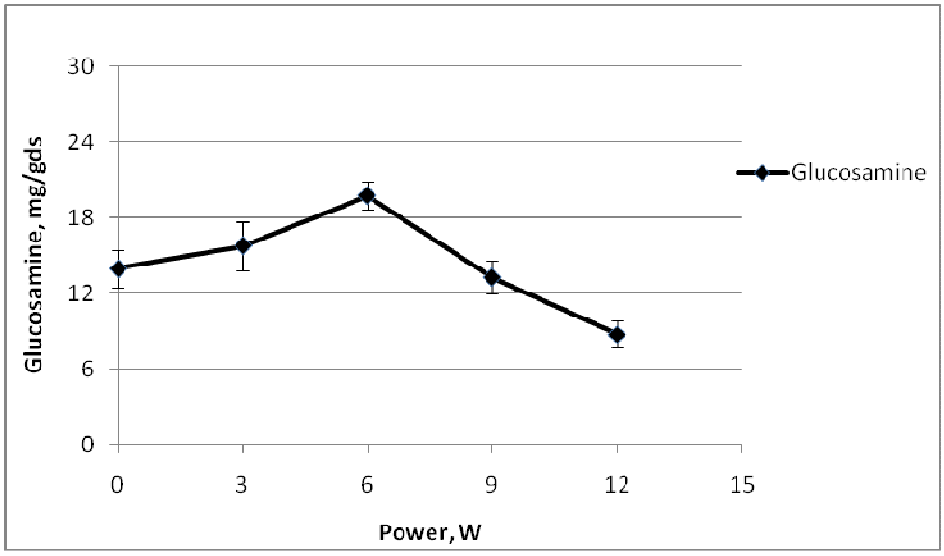


Figure 8.10 Change in the glucosamine concentration of angkak made from ground adlay after the exposure of the culture to ultrasound of 1.080 MHz and different powers for 10 minutes per day for three weeks.

Figure 8.11 shows pigment development and moisture content when daily exposure to ultrasound was operated for 5 minutes. The moisture content now only decreased slightly when a power of 12 Watts was used. The pigment production increased from 0 to 9 Watts to reach a maximum and then decreased; again this coincides with a loss of moisture.

Figure 8.12 shows the change in glucosamine concentrations. The peak in the glucosamine production again coincided with the peak in the pigment production. The pigment was homogeneously distributed in the sample obtained at 9 Watts.

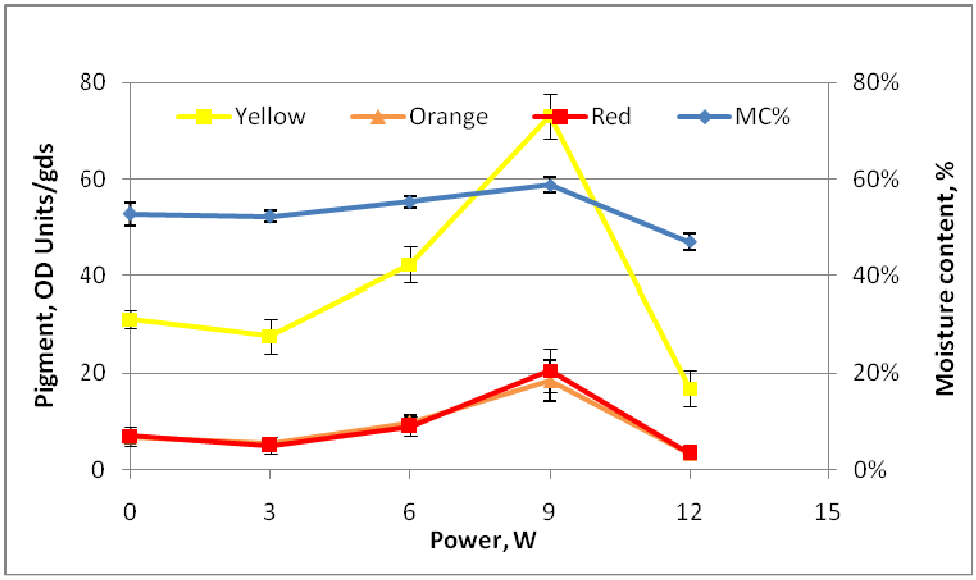


Figure 8.11 Study of the effect of ultrasound power on a culture of *Monascus* on ground adlay. The ultrasound had a frequency of 1.080 MHz, and the exposure time was 5 minutes per day for three weeks.

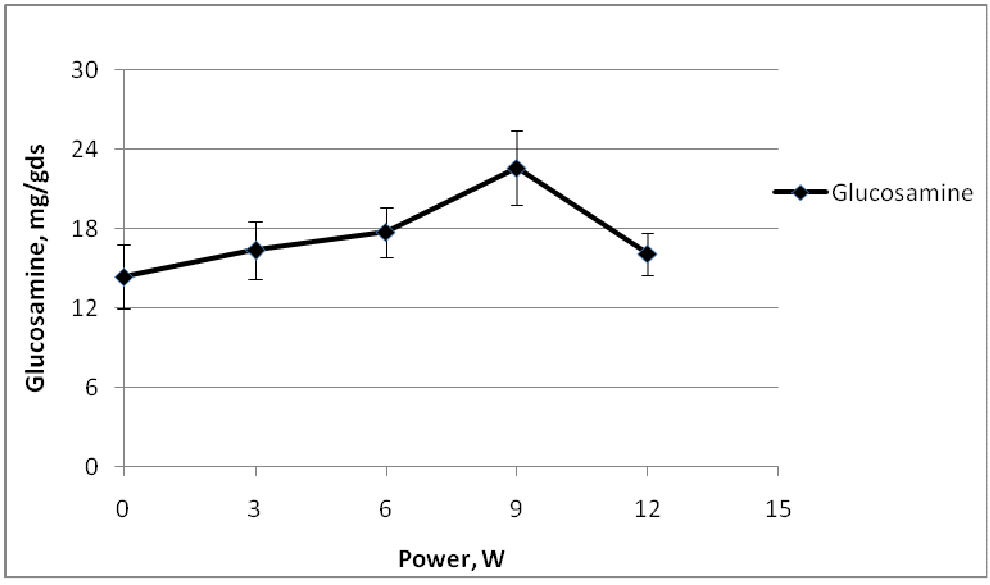


Figure 8.12 Change in the glucosamine concentration of angkak made from ground adlay after the exposure of the culture to ultrasound of 1.080 MHz and different powers for 5 minutes per day for three weeks.

8.3 Conclusions

The studies carried out with *Monascus* confirmed that ground adlay had severe limitations as a substrate for solid state fungal fermentation compared to whole grain adlay. This was due to the greater stickiness of the cooked material, limiting oxygen transfer into the bed and thereby limiting oxygen transfer.

The application of audible sound and ultrasound could, to some extent, improve this situation. Audible sound was the least successful. Sound vibration could easily move non-sticky particles but the sticky material formed after cooking would have needed more highly powered vibrations to move the particles. The large forces of vibration needed might well have caused the cells to break up. A small daily dose of ultrasound proved more successful. A more homogeneous product was obtained when the bed material was exposed to ultrasound. Under optimal conditions (daily exposure to ultrasound of approx. 1 MHz) for a brief period of time depending on relatively low power levels, *Monascus* pigment concentration could be increased 2 times compared to the static culture (untreated sample).

CHAPTER 9

BIOMASS AND PIGMENT PRODUCTION BY *MONASCUS* DURING MINIATURE SUBMERGED CULTURE ON ADLAY

9.1. Introduction

Angkak is a natural red colourant which is usually made by culturing *Monascus spp*. on cooked rice. *Monascus* can produce coloured pigments which are yellow (monascin and ankaflavin), orange (rubropunctatin and monascorubrin) and red (rubropunctamine and monascorubramine) (Chiu and Poon, 1993). In general, pigment production can be divided into two processes: solid state and submerged culture (Dufossé *et al.*, 2005; Jůzlová *et al.*, 1996 and Wang *et al.*, 2007). Pigment production during submerged culture is more uniform than in solid state culture but product concentration is much lower and much more waste water is produced (Hölker *et al.*, 2004 and Singhania *et al.*, 2009). Solid state fermentation on the other hand is much more laborious. Clearly more work is needed to determine the relative benefits of submerged and solid state culture.

Culture times of *Monascus* are often long, typically several weeks, and optimization of a production process can therefore take a long time. To reduce the time needed for optimization, Miniature Bioreactors (MBRs) could be used. MBRs can be classified into shaken devices (flask, microtitre and spin tubes), stirred fermenters, bubble columns and other miniature devices (Betts and Baganz, 2006, Duetz, 2007; Hessel *et al.*, 2008; Lamping *et al.*, 2003, Micheletti and Lye, 2006, Weuster-Botz *et al.*, 2001 and Weuster-Botz *et al.*, 2002). These bioreactors typically have a small volume, ranging from ca. 0.1 mL to approx. 100 mL. Their use can reduce labour and material costs. In addition, many cell cultures can be performed in parallel and high efficiencies can be achieved in agitation and aeration. This increases the amount of oxygen transfer and in turn increases the amount of product that oxygen-dependent bioprocesses can yield. It can be expected that growth kinetics and product formation at miniature-scale can be scaled up quantitatively.

Currently, high power monitoring tools are available that enable biochemical engineers to understand and explain quantitatively the presence and activity of cells and the production of their metabolites during cultivation (Stockar *et al.*, 2003). Such methods can be of considerable help during scale up and scale down of fermentation processes. The presence of fungal biomass is a requisite for pigment production, and a strong relation can therefore be expected between biomass levels and pigment production. Measurement of biomass is difficult, especially on-line and in media with high concentrations of particulates which can interfere. Capacitance measurements are one of the best methods for measuring cell concentrations. The success of the method depends on the large difference in the electrical properties of the membrane (dielectric constant or permittivity and conductivity), the cytoplasm and the suspending medium (Fehrenbach *et al.*, 1992, Mantanguihan *et al.*, 1994, Mishima *et al.*, 1991 and Peñaloza *et al.*, 1991). This results in a change in the capacitance from low to high frequencies in the frequency range 100 kHz - 100 MHz, which can be can be related directly to the microbial biomass concentration. Measurement of the capacitance of a cell suspensions at a single low frequency (typically around 400 kHz), or better the difference between a low and high frequency, can be used to overcome the need for other off line and often time-consuming methods for biomass measurement. An added advantage is that it only measures the volume fraction of biomass that is enclosed by a cellular membrane. Thus, the presence of non-cellular material without a membrane (such as rice or adlay) does not have a major influence on the measurement, and cell concentrations can be measured in the presence of high concentrations of other materials (Peñaloza *et al.*, 1991).

In one of the previous chapters, it was shown that solid state culture of *Monascus* culture on whole grain and puffed adlay is possible. An attempt to use ground adlay was however unsuccessful because *Monascus* failed to penetrate deep into the bed material and remained near the top of the bed material. The reason for this was the fact that the grains were sticky and gelled together to form a mass without voids for air to penetrate. The application of ultrasound helped but did not overcome the problem. In this chapter, it is attempted to produce adlay angkak by the submerged culture on ground adlay. It is expected that the oxygen transfer in this system will be better than in the solid state culture because the liquid can be aerated and mechanically stirred, and conversion fast because of the large surface area of the ground adlay. Different reactor

types are explored, including miniaturised bioreactors, and combined with on-line monitoring to investigate biomass levels.

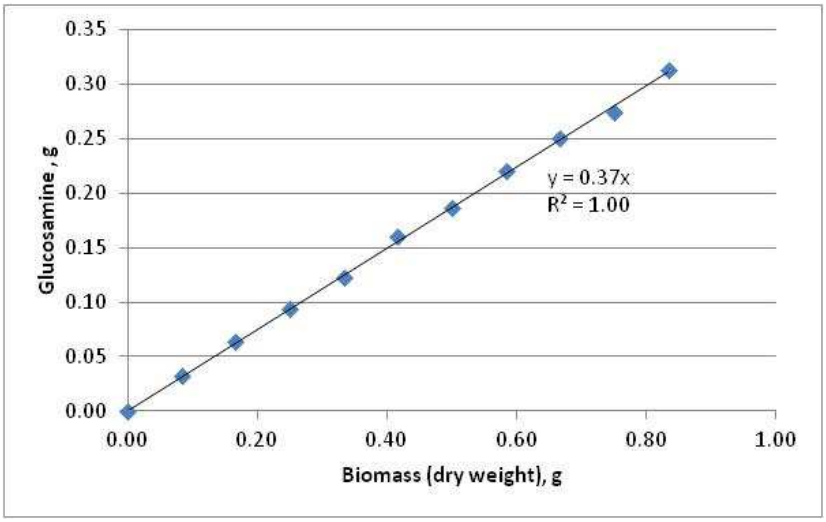
9.2. Results and discussion

9.2.1 Calibration of the relation between fungal biomass, glucosamine concentration and capacitance signal

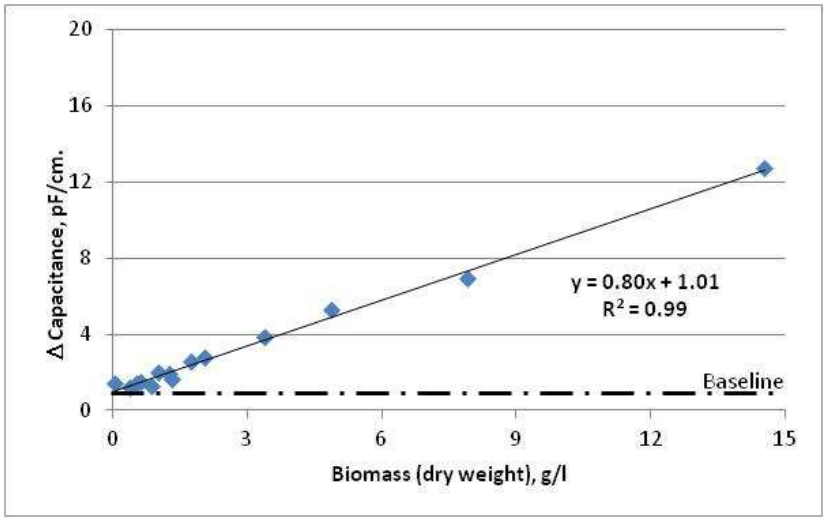
The relationship between capacitance and the cell concentration was determined first. To calibrate the Biomass Monitor, *Monascus* was cultured in five magnetically stirred Erlenmeyer flasks with 100 mL 4% D-glucose solution for seven days. The samples were harvested by centrifugation to obtain a suspension with a high concentration of fungal biomass. A 100 mL mineral solution was prepared. The concentrated fungal biomass was added stepwise to the stirred beaker with the mineral solution and the capacitance signal measured in the range 100 kHz - 20 MHz (see 3.7.4 in Chapter 3). Samples were also taken from the beaker to obtain the dry weight, and the glucosamine content of the samples (see 3.7.5 in Chapter 3).

The correlation between the dry weight of cells and glucosamine content is shown in Figure 9.1(a). A linear relation between the fungal dry biomass weight and glucosamine content can be observed described by the equation: $Y= 0.37x$. The relation between the biomass in dry weight and the Δcapacitance at 465 kHz - 20 MHz is shown in Figure 9.1(b). A linear relation can be seen between biomass (dry weight) and Δcapacitance described by the equation: $Y= 0.80x +1.01$. The correlation between the dry weight of cooked adlay and the Δcapacitance at 465 kHz - 20 MHz is shown in Figure 9.1(c). A linear relation can be expressed in the equation: $Y = 0.02x + 1.07$. The capacitance signal from adlay at the same concentration is therefore 40 times lower than the capacitance signal from fungal biomass.

(a)



(b)



(c)

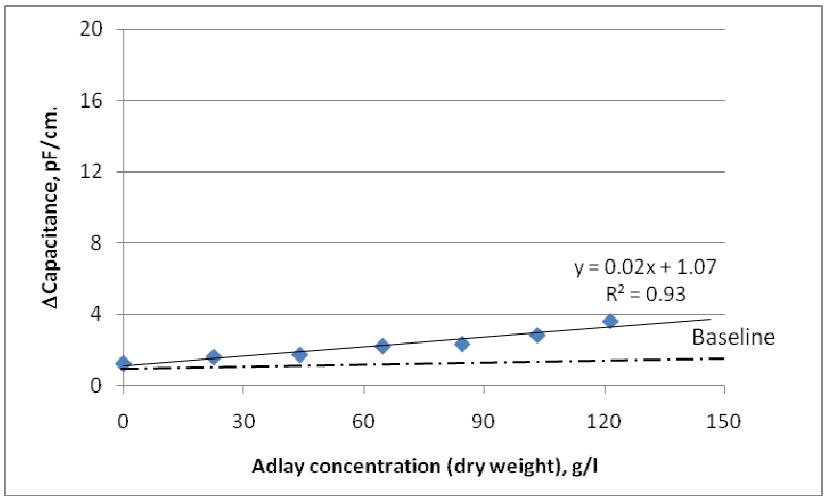


Figure 9.1 Biomass and substrate calibration

(a) Relation between dry weight and glucosamine content.

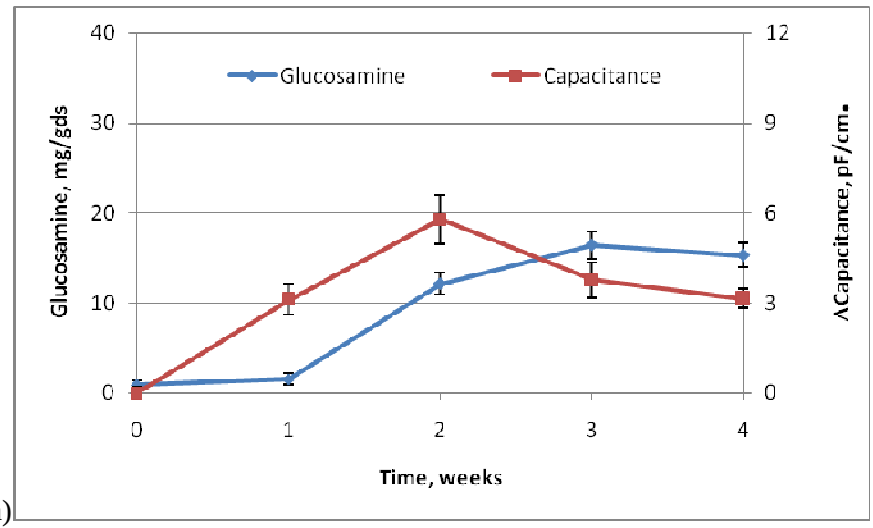
(b) Cell dry weight and ΔCapacitance between 465 kHz - 20 MHz

(c) Adlay dry weight and ΔCapacitance between 465 kHz - 20 MHz

9.2.2 Adlay submerged culture in shaken flasks

Figure 9.2 shows the changes in the glucosamine and pigment concentration and capacitance during a shaken flask culture during four weeks of fermentation (see 3.4.2.1 in Chapter 3). The Δcapacitance value at 465 kHz - 20 MHz reached a maximum value of 5.8 ± 0.8 pF/cm in two weeks; however the glucosamine concentration kept increasing until week 3. Pigment production was small in the first week and reached its maximum after three weeks. A drop in the capacitance at the later stages of batch fermentations is commonly found and is thought to be caused by a drop in the viability of the cells, the capacitance of nonviable cells being much lower than that of viable cells (Fehrenbach *et al.*, 1992, Matanguihan *et al.*, 1994, Mishima *et al*., 1991 and Penaloza *et al.,* 1991). Glucosamine content, however, is indicative of the presence of cell wall material only, and not dependent on viability. Even though less viable, the cells still continued pigment production. The pigment content therefore kept increasing even though the capacitance declined. Interestingly, the decline in capacitance coincided with a decrease in the rate of pigment production, indicating a possible role of capacitance measurements in indicating when the rate of pigment production will decline.

(a)



(b)

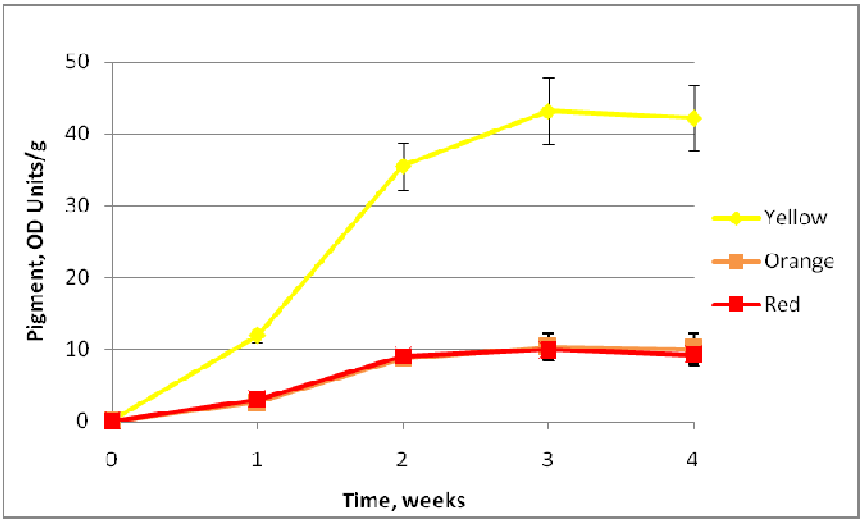


Figure 9.2 Submerged fermentation in shaken flasks

(a) ΔCapacitance and glucosamine concentration

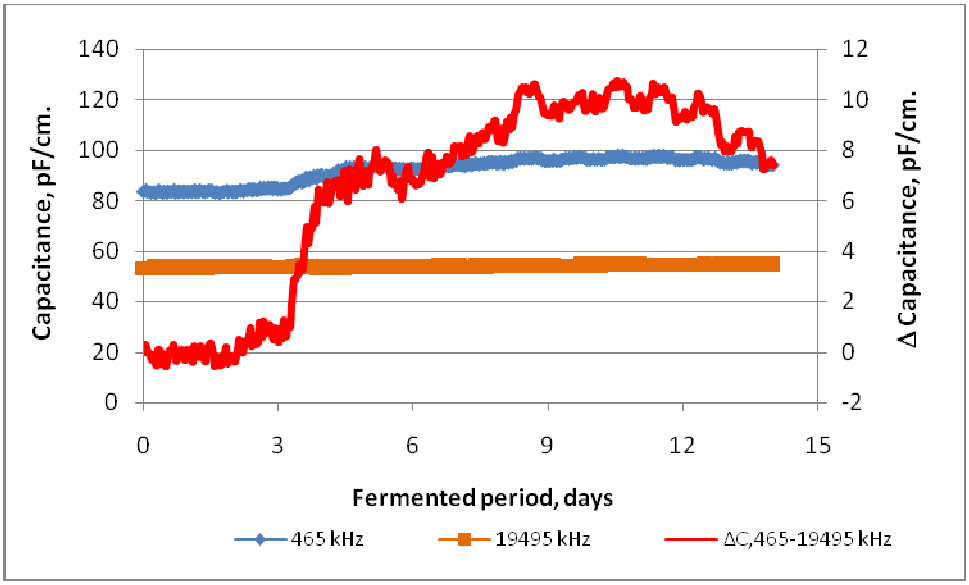
(b) Pigment production

9.2.3 Comparison of *Monascus* growth and pigment production in shaken and stirred reactors

In the next set of experiments, a comparison was made of the culture of *Monascus* in a shaken (150 rpm) and a stirred (800 rpm) miniaturized fermenter. In both types of reactors, the biomass was monitored on-line using capacitance measurements. After the culture had finished, samples were taken to measure the pigment and glucosamine concentrations. It was not possible to take samples during culture.

Changes in the capacitance at low and high frequencies, and the difference between the two frequencies, during the fermentation in the two reactor types are shown in Figure 9.3 (see 3.4.2.4 in Chapter 3). In the shaken miniaturized bioreactor in the first 3 days, the Δcapacitance signal only increased slightly. After that, it increased rapidly to reach the maximum value in 12 days. After 12 days, the signal started to drop off. In the stirred miniaturized bioreactor the lag phase was much shorter (a day) and the increase in capacitance much faster than in the shaken reactor. A maximum in the Δcapacitance was reached after a period of only six days and decreased after eight days.

(a)



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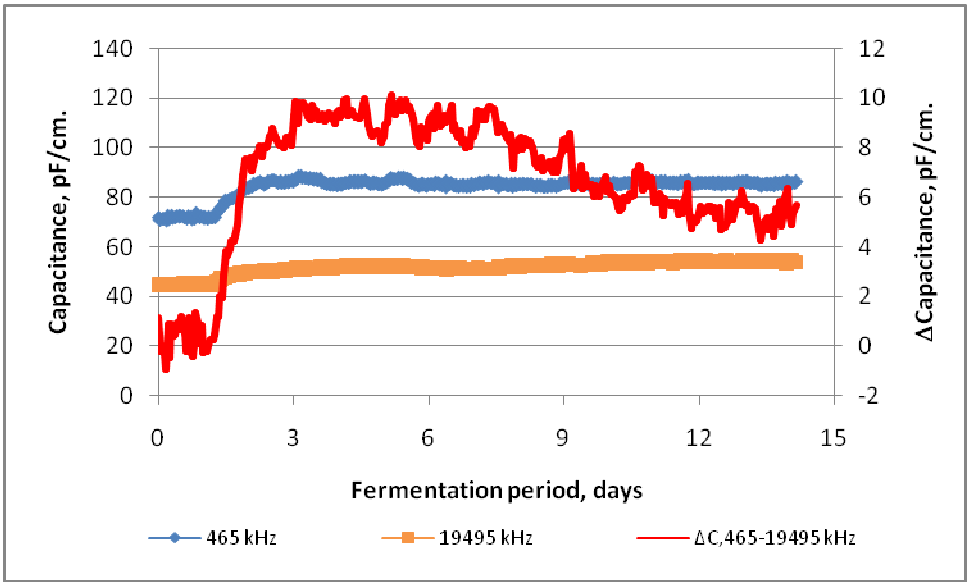


Figure 9.3 Capacitance signal during submerged culture of *Monascus*
(a) Shaken bioreactor (b) Stirred bioreactor

In Figure 9.4, the results of the chemical analysis of the product of the fermentations are shown. When comparing both of the miniaturized systems, the total pigment and glucosamine content of the material is found to be higher in the stirred reactor in the shaken reactor column. The pigment composition in the shaken reactor is also different from that in the stirred reactor, with more orange and red pigment being produced in the stirred reactor than in the shaken reactor. The appearance of extracted pigment from the shaken reactor was distinctly yellow, whereas from the stirred reactor it was red.

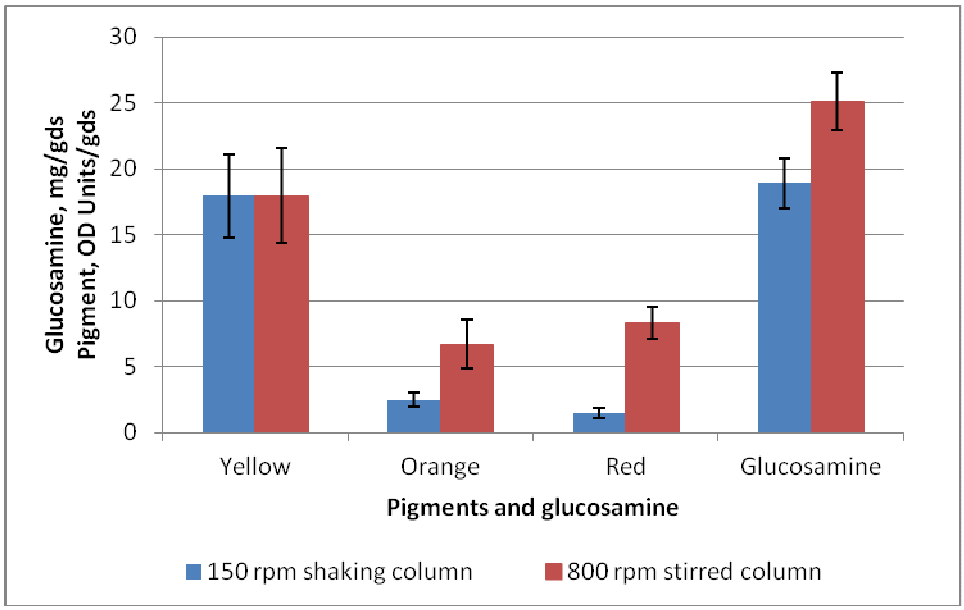


Figure 9.4 Comparison of *Monascus* pigment and glucosamine concentration in ground adlay after two weeks of culture in shaken and stirred bioreactors

The difference in the results is arguably because there is a greater transfer of oxygen in the stirred reactor than in the shaken reactor (Kloeckner and Buchs, 2012, Micheletti and Lye, 2006). The glucosamine concentration and the capacitance levels in the two systems were not significantly different. This means that the oxygen content in miniature scale reactors may be sufficient to create biomass, but in the case of the shaken reactor not sufficient to realise *Monascus'* fullest potential in pigment production, presumably because the production of secondary metabolites has a higher oxygen demand than the primary metabolism needed for biomass production.

9.2.4 Effect of the rotating speed of the impeller and the volume of liquid on the culture of *Monascus* in a miniaturized bioreactor.

To investigate this further, the effect of the rotating speed of the impeller and the volume of liquid in the reactor on biomass and pigment production was studied. It can be expected that an increasing rotating speed increases oxygen dispersal, and that an increasing volume increases oxygen demand. Rotating speeds were varied between 400, 800 and 1200 rpm, and the volume of liquid in the reactor medium from 10 to 30 mL. The duration of the culture was seven days. Figure 9.5 shows the effect of the impeller speed and the volume of the liquid in the reactor on glucosamine production. The expectations mentioned above were confirmed, i.e. a higher stirred speed led to a higher glucosamine concentration, and increasing the volume in the reactor decreased it. Interestingly, at higher rotating speeds the effect of volume of liquid in the reactor on the glucosamine production was less than at lower rotating speeds. This may indicate that mixing is more highly efficient at higher speeds.

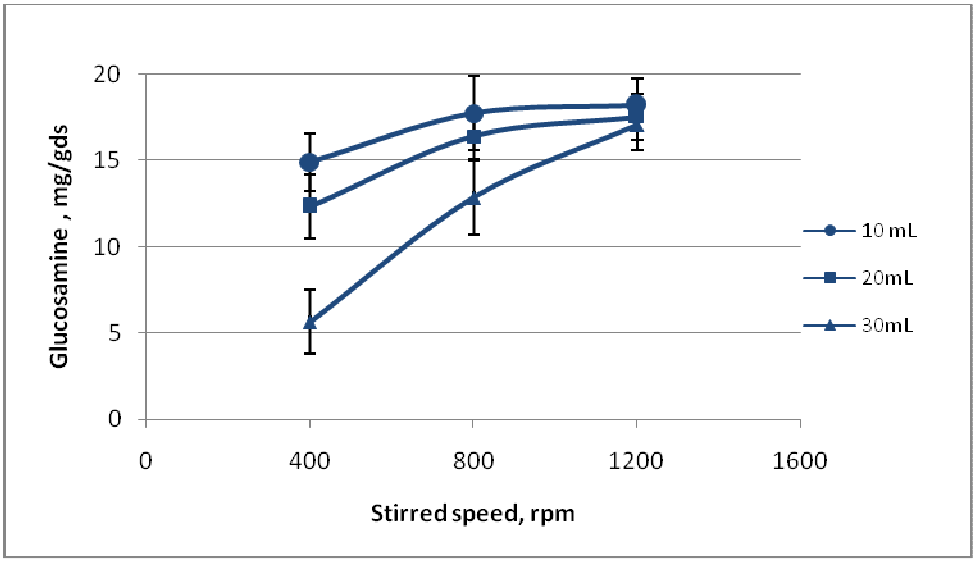


Figure 9.5 Glucosamine production in a stirred miniature submerged culture of *Monascus* on ground adlay

Figure 9.6 shows the effect of the impeller speed and the volume of the liquid medium in the reactor on the Δcapacitance after a seven day fermentation period. As expected, the data shows a positive effect of the rotation speed of the impeller and a negative effect of the volume of the liquid medium in the reactor. However, unlike the previous results obtained with glucosamine, the Δcapacitance signal at the higher impeller speed of 1200 rpm was different for different volumes in the reactor. This may arguably be because the shear in the bioreactors is different. Shear affects cell viability and hence capacitance (Markx *et al.*, 1991). Shear is known to damage *Monascus*

filaments and change their morphology, and affect pigment production (Kim *et al.*, 2002, Wang *et al.*, 2003 and Wang *et al.*, 2007). The higher shear stress at higher impeller rates may have affected the cell viability in different ways at different volumes in the reactor. Kim *et al.* (2002) showed that a maximum tip speed of 2.0 m/s could be used in their fermentation of *Monascus*. When they used higher tip speeds, the pigment production dropped because of excessive shear. The maximum tip speed used in these experiments in stirred miniaturised bioreactors was 1.3 m/s. The stirrer speed was therefore below the maximum, and could potentially be increased.

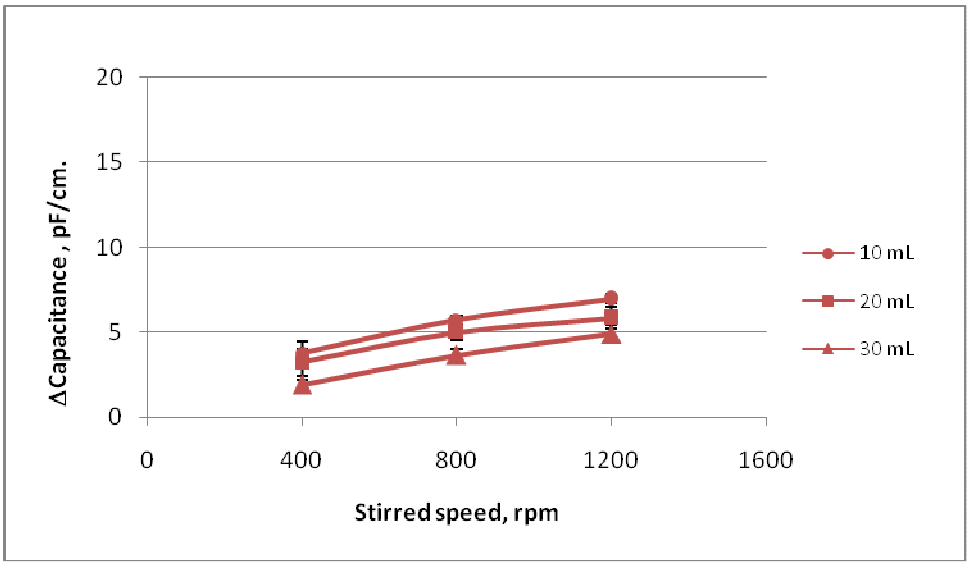
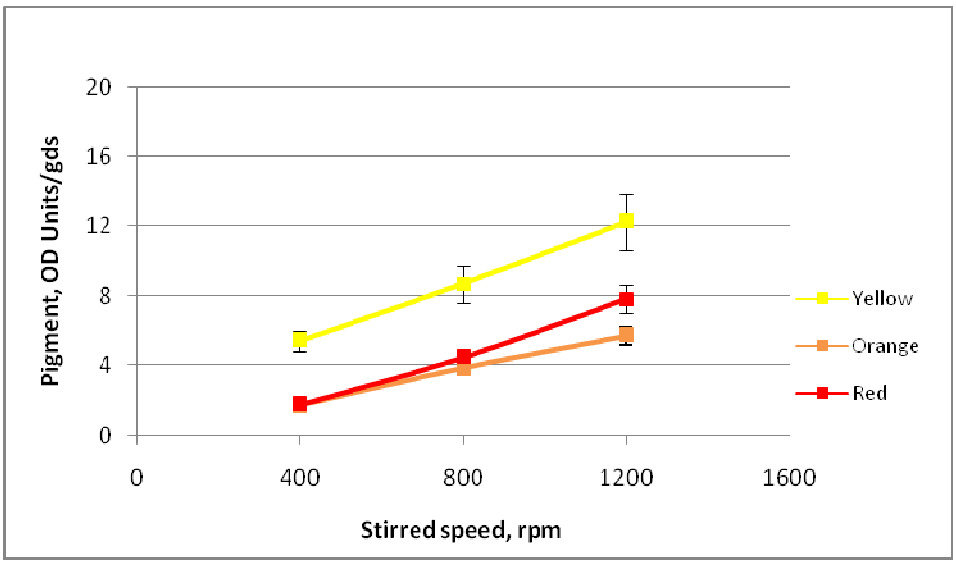


Figure 9.6 Capacitance signal in a stirred miniature submerged culture of *Monascus* on ground adlay

Figure 9.7 shows the effect of the impeller speed and the volume of liquid in the reactor on the pigment yield in the miniaturized bioreactor. As expected, the rotating speed had a positive effect and liquid volume a negative effect. In the 10 mL fermenter, the pigment production increased more than 2.5 times within the seven days of culture when the rotating speed was increased from 400 rpm to 1200 rpm. In the 20 mL fermenter, the pigment concentration increased less than in the 10 mL fermenter when operated with the same rotating speed. Finally, in the 30 mL fermenter, the pigment yield increase with the impeller speed was similar to that in the 20 mL fermenter. The highest pigment yields were achieved in the smallest volume and at the highest impeller speed. Significantly more red pigment was produced in the smaller volume and at the higher impeller speed, again indicating that more oxygen is needed for the red pigment production.

(a)



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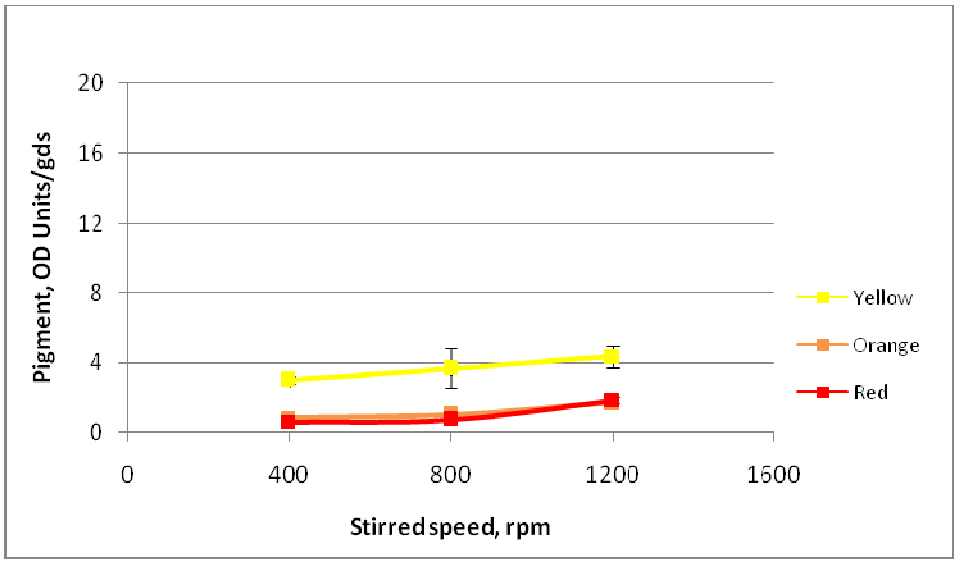

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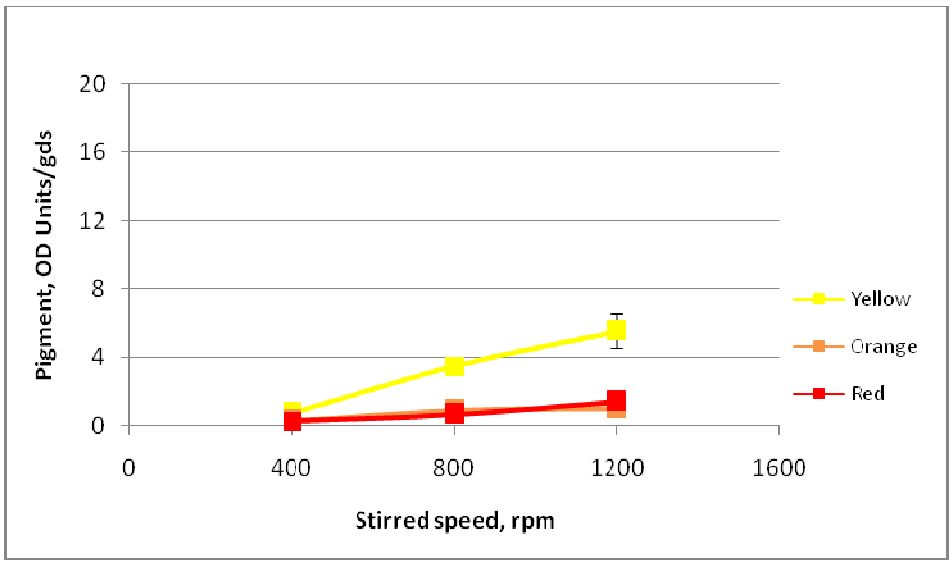

Figure 9.7 *Monascus* pigment production in a stirred miniature submerged culture on ground adlay

(a) 10 mL miniature reactor (b) 20 mL miniature reactor (c) 30 mL miniature reactor

9.3 Conclusions

Miniaturized bioreactors were successfully used to investigate pigment and biomass production during submerged fermentation of *Monascus* on adlay. Shake flasks gave the highest pigment concentrations, but on-line measurements were not possible, and scale up is difficult. Miniaturized shaken bioreactors were developed in which continuous biomass measurements using dielectric spectroscopy were possible, but pigment production was less successful, most likely due to poor mixing and oxygen transfer in the shaken column being lower. Stirred bioreactors proved better, with better pigment yields at smaller volumes of liquid in the fermenter at higher impeller speeds again indicating that the availability of oxygen is a determining factor in pigment production.

Dielectric spectroscopy proved to be an effective method for measuring biomass continuously in the miniaturized reactors. It also gave useful information about cell viability. Extension of the number of sensed parameters during the miniaturized submerged culture of *Monascus*, for example by the use for sensors for oxygen or pigment concentration, could increase the amount of information available even further.

CHAPTER 10

CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

In this thesis, an investigation is described as attempts to use adlay as an alternative substrate for the culture of *Monascus* for the production of adlay angkak. Angkak can be used as a natural colouring agent and also contains statins which can reduce cholesterol levels; the use of adlay could give a product with lower concentration of the mycotoxin citrinin than other substrates.

Three different forms of adlay were used: whole grain adlay, puffed adlay and ground adlay. Different fermentation methods were also used. Whole grain adlay and puffed adlay were successfully used in solid state fermentations in tray bioreactors. Both materials leave voids between the particles which allow air to ingress into the bed. Puffed adlay was best. Puffed grains have a higher porosity than normal grains. Although particles of puffed adlay shrink when they are cooked in the autoclave, some of the porosity remains. As a result, the material is more easily digested, transport processes inside the particles are faster and penetration of the fungus into the particles is rapid. Puffed grains rapidly take up water, and the water is distributed evenly across the particle. The material is also less sticky. Ground adlay was least suitable for solid state fermentation. In theory, the fermentation with fine particles should be faster because the surface area is larger. However, the ground material was very sticky when cooked, and no voids were left between the particles after cooking. This caused oxygen transfer limitations to occur rapidly in a culture on ground adlay, limiting fungal culture to a small layer at the top.

For product development, it is important to make the yield of pigment as high as possible. Mixing nutrient supplements into the substrate of solid state fermentations was shown to be a relatively easy way of enhancing the pigment yield. The addition of histidine was most effective; it increased the pigment yield on adlay (Chinese pearl barley) more than monosodium glutamate or nitrate or sugars. The addition of histidine could also help to reduce citrinin levels. In puffed adlay optimisation of the amounts of histidine, fructose and moisture added to the substrate helped to increase the pigment yield by more than 10 times.

Adding pure histidine increases the production cost. In future experiments, histidine from natural sources such as soy protein isolate could be mixed with adlay grain to reduce the cost. Though not investigated here, the addition of fatty acids as nutrient supplements may also be helpful. Octanoic acid is a medium chain fatty acid which has been successfully added to the glucose medium, increasing the pigment production by 2 times (Hajjaj *et al.*, 2000).

Co-culture of *Monascus* and yeast cannot be recommended for increasing pigment production by *Monascus* when grown on adlay. Co-culture *Monascus* with yeast entails a significant amount of additional effort, yet it only slightly increased pigment production.

Puffed adlay was also found to be good material for the culture of *Monascus* in rotating drum bioreactors. Common whole grains were less suitable as they had a higher stickiness and grains tended to stick together and also to the chamber wall. Continuously rotating bioreactors in miniature scale were successful with up to 80% of single particles but the pigment yields were low. An intermittently rotating bioreactor, where the bioreactor was only rotated once or a few times a day for a short period gave a lower percentage of single particles but a higher pigment yield. In theory, a high rotating speed can solve heat and mass transfer problems and give product homogeneity (Bhargav *et al.*, 2008) but fermented products can be aggregated into balls (Pandey, 1991). Furthermore, the high shear rates involved can have a negative effect on product yields (Mitchell *et al.*, 2006, Pandey, 1991 and Saucedo-Castaneda *et al.*, 1992). Intermittent rotation is therefore better.

The use of alternative types of reactor for solid state fermentations such as paddle mixers and packed beds or fluidized beds with forced aeration were not attempted here, but could be in future work. As mixing with a paddle was expected to damage the cells and lead to low product yields, it was not attempted. Because of the sticky nature of the adlay a fluidised bed was expected to be impractical. A packed bed with forced aeration could have been tried. Preliminary experiments, however, showed that control of the moisture levels in the bed was difficult and moisture distribution in the bed tended to become uneven.

Sound and ultrasonic vibration were applied to increase the pigment production on the ground adlay. Because of the stickiness of the material, the aggregated particles of ground adlay are difficult to move with audible sound. The pigment production only increased slightly with a strong vibration at 40 Hz. A short dose of low intensity high frequency (1 MHz) ultrasound did increase the *Monascus* pigment yield. The pigment was also distributed more evenly in the matrix. At a high dose of ultrasound, fungal cells were killed and the material had no pigment. This was possibly due to the shear forced produced by the ultrasound, but heat produced by the transducer may have been a contributing factor. The most successful treatment with ultrasound vibration involved using 1 MHz and 9 Watts in 5 minutes daily. This increased the pigment concentrations twice and gave a homogenous product.

Submerged culture was studied as an alternative method for the fermentation of ground adlay. A submerged culture on ground adlay in a shaken bioreactor gave a relatively low biomass and pigment yield due to poor mixing and oxygen transfer. A miniaturized stirred fermenter proved much better than a shaken bioreactor and gave a higher biomass and pigment yield. Even then, oxygen transport was still a major factor in limiting biomass and pigment production. When the speed of the impeller was increased, biomass and pigment production also increased. When the level of liquid volume was changed, a better pigment yield was found with smaller volumes of liquid in bioreactors at higher impeller speeds. At higher rotating speeds, the effect of the volume of liquid in the reactor on the biomass production was less, indicating that the oxygen concentration in the miniature scale bioreactors may be sufficient to create biomass but not sufficient to reach the cells' full capacity for pigment production. Increasing the stirrer speed, however, may cause an undesirable effect on cell growth and pigment production due to high rates of shear. This should be investigated further, as well as the use of the different types of impeller.

A dielectric biomass sensor was successfully used in submerged fermentations on ground grain. The use of the biomass sensor in solid state fermentation could be explored further. Other sensors could also be added, both in submerged and solid state fermentations. An oxygen sensor may be particularly useful to determine the availability of oxygen. Humidity and moisture sensors could also be extremely useful, in particular to measure and control moisture in the substrate bed during solid state fermentations.

Further work could be done on product and process development. In particular, research could be done on modifying the ground adlay particles to reduce stickiness and increase oxygen diffusion. For example, it could be investigated whether or not ground puffed adlay could be created. Culture of *Monascus* on this modified ground grain may be more successful than on ground grain due to the particles potentially having a high porosity, less stickiness and lower density. If such a non-sticky fine material can be made then the use of ultrasound to increase product homogeneity could be explored again. Also, a vibration belt conveyor fermenter could be used, possibly combined with high frequency ultrasound to expose temporarily the bed to ultrasound and mix the material, to produce pigment in a continuous process. Alternatively, ground puffed adlay could be used in a submerged culture. The high porosity would ensure the material is converted more quickly.

Overall, it could be concluded that puffed adlay is superior to whole grain adlay for pigment production because it can be used to make a product with a high concentration of pigment faster. Also, the material is less sticky and it lends itself for use in the solid state cultures in which the bed of substrate is mixed such as the rotating drum bioreactor, giving a more even production process and a more homogenous product. It is still uncertain as to whether solid state fermentation or submerged fermentation of adlay is better. Stickiness of the substrate is less of a problem in submerged fermentation, and homogeneity is almost guaranteed. Moisture levels in the substrate are not an issue. The forced aeration in submerged culture is much easier than in solid state culture, though ensuring that enough oxygen is transferred into the liquid is still a potential problem, as is the shear sensitivity of the cells. At a large scale, oxygen diffusion will also become limiting in the submerged culture where in the solid state fermentations problems at the large scale can be expected with heat accumulation and oxygen diffusion. Further work still needs to be done on this. Monitoring is easier in submerged culture than in solid state cultures. However, nowadays, there are many high power monitoring tools which can quantitatively measure the presence and activity of cells and the production of the metabolites during solid state cultivation (Stockar *et al.*, 2003). A solid state culture has a lower water demand, less waste generation, higher product stability and high production per reactor volume than a submerged culture. Some of these problems can however be resolved by higher substrate concentration – provided, of course, that oxygen transport and shear do not become an issue.

CHAPTER 11

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